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(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 26 November 2003 (26.11.03)	
Applicant's or agent's file reference UNI-004-PCT	IMPORTANT NOTIFICATION
International application No PCT/EP03/11192	International filing date (day/month/year) 09 October 2003 (09.10.03)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 09 October 2002 (09.10.02)
Applicant UNIBIOSCREEN S.A. et al	

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Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
09 Octo 2002 (09.10.02)	PCT/EP02/11310	EP	26 Nove 2003 (26.11.03)

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PCT/EP 03 / 11192

Bescheinigung

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten internationalen Patentanmeldung überein.

Certificate

The attached documents are exact copies of the international patent application described on the following page, as originally filed.

Attestation

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet international spécifiée à la page suivante.

Den Haag, den
The Hague,
La Haye, le 21. 11. 2003

Der Präsident des Europäischen Patentamts
Im Auftrag
For the President of the European Patent Office
Le Président de l'Office européen des brevets
p.o.

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PRIORITY DOCUMENT
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Patentanmeldung Nr.
Patent application no.
Demande de brevet n° PCT/EP 02/11310

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Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.: PCT/EP 02/11310
Application no.:
Demande n°:

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Bezeichnung der Erfindung: Extract with anti-tumor and anti-poisonous activity
Title of the invention:
Titre de l'invention:

Anmeldetag: 09 October 2002 (09.10.2002)
Date of filing:
Date de dépôt:

In Anspruch genommene Priorität(en)
Priority(ies) claimed
Priorité(s) revendiquée(s)

Staat:	Tag:	Aktenzeichen:
State:	Date:	File no.
Pays:	Date:	Numéro de dépôt:

Benennung von Vertragsstaaten : Siehe Formblatt PCT/RO/101 (beigefügt)
Designation of contracting states : See Form PCT/RO/101 (enclosed)
Désignation d'états contractants : Voir Formulaire PCT/RO/101 (ci-joint)

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PCT REQUEST

UNI-004-PCT

Original (for SUBMISSION) - printed on 09.10.2002 03:11:54 PM

V	Designation of States	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE BG CH&LI CY CZ DE DK EE ES FI FR GB GR IE IT LU MC NL PT SE SK TR and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ OM PH PL PT RO RU SD SE SG SI SK SL TJ TM TN TR TT TZ UA UG US UZ VC VN YU ZA ZM ZW
V-5	Precautionary Designation Statement In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	Exclusion(s) from precautionary designations	NONE
VI	Priority claim	NONE
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)

Extract with anti-tumor and anti-poisonous activity

Field of the invention

The present invention relates the medical field. The invention relates in a first aspect to an extract of the plant *Calotropis procera,* having a pharmacological activity, in particular an anti-tumor activity and/or anti-poisonous activity and active compounds isolated thereof. In a second aspect, the present invention relates to a method for obtaining said extract. The invention further relates in a third aspect to a pharmaceutical composition for the treatment of cancer comprising an effective amount of said extract or an active compound thereof. In a fourth aspect, the present invention concerns the use of said extract or an active compound thereof as a medicament and the use of said extract or an active compound thereof for the preparation of a medicament for the treatment of cancer.

Background of the invention

Cancer develops in a given tissue when some genomic mutation perturbs cell cycle kinetics by increasing cell proliferation or decreasing cell death, or both. This perturbation leads to unrestrained growth of a genomically transformed cell population. Some cells from this transformed cell population may switch to the angiogenic phenotype, enabling them to recruit endothelial cells from the healthy tissue and leading to the sustained growth of the developing neoplastic tumor tissue. Subsequently, some cells migrate from the neoplastic tumor tissue and colonize new tissues, using blood or lymphatic vessels as major routes of migration. This process is also known as the metastatic process.

In practice, most of the agents used today in hospitals to treat cancer patients are drugs, which more or less directly target the cell kinetics, i.e. cell proliferation, of the cancer to be combated. The working mechanism of such anti-cancer drugs essentially relates to the disruption of the development of malignant cells by acting on cell kinetics. These drugs include alkylating agents, intercalating agents, antimetabolites, etc.., most of which target DNA or enzymes regulating the DNA duplication and elongation process. These drugs attack DNA.

A major drawback of these drugs involves that the drugs do not work in a selective manner, i.e. they do not select between normal and neoplastic cells. They are used in accordance with the fact that the DNA of rapidly proliferating cells, i.e. cancer cells, is more sensitive to this

type of agents than the DNA of less rapidly proliferating cells, i.e. normal cells. However, rapidly growing tumors are not always tumors exhibiting high levels of cell proliferation. Rapidly growing tumors may also include tumors which exhibit low levels of cell death compared to the normal cell population from which these tumor cells issue. For these types of rapidly growing tumors, the mentioned drugs are not effective.

In addition, the great majority of the drugs used in the standard treatment of cancer using the cell kinetics approach have the drawback of being toxic or even highly toxic, i.e. involving many detrimental side-effects on healthy cells, tissues and organs, and this limits their clinical use to a relatively low number of administrations per patient. In addition, several of these compounds must be combined into a poly-chemotherapeutic regimen in order to have any observable effect against cancer. By way of evidence such anti-cancer drug combinations increase detrimentally the toxicity of the treatment and also limit the number of administrations that can be applied.

Some anti-cancer drugs from natural origins, such as e.g. anti-tubulin compounds, using a therapeutic approach different from the cell kinetics approach, have been proposed. Said drugs aim to prevent the migration of cancer cells which escape from the tumor bulk and first invade neighboring tissue therefore establishing metastases. However, the compounds of this type known so far also show major toxic side-effects, which limits their use over long periods of treatment.

Therefore, there remains an urgent need in the art for finding improved anti-cancer drugs, which overcome at least some of the above-mentioned drawbacks. Consequently, it is a general object of the invention to provide improved anti-cancer drugs. In particular the invention aims to provide an improved anti-cancer drug, showing minimal side effects.

Summary of the invention

The present invention relates to an extract of the plant *Calotropis procera*. In a first embodiment the invention relates to an extract of the plant *Calotropis procera,* which has a pharmacological activity, in particular an anti-tumor activity and / or an anti-poisonous activity. Surprisingly, the extract according to the invention combines an anti-tumor effect with an anti-poisonous activity.

According to the present invention the term "anti-tumor activity", refers to the *in vitro* as well as *in vivo* anti-tumor effects exerted by the extract or isolated compounds thereof. The anti-tumor effects essentially include but are not limited to a dramatic decrease of cell growth and a pro-apoptotic effect. Importantly, the extract according to the invention exhibits anti-tumor activity on a large number of cancer types, such as breast cancer, melanoma or lymphoma cancers amongst others.

Another feature of the extract according to the invention encompasses its anti-poisonous activity. The term "anti-poisonous activity" refers to the ability of the extract according to the invention or isolated compounds thereof to attenuate or reverse the effects of poisonous compounds. A "poisonous compound", as used herein, refers to a compound that exerts a relevant detrimental, toxic effect(s) on normal, i.e. non-cancer related cells, tissues or organs. The term poisonous compound may thus also include a compound, drug or medicament, used to treat a particular disease that exerts detrimental toxic side effects.

In another embodiment, the present invention relates to the active compounds, isolated from the extract. The term 'active compounds' or 'active components' of the extract are used herein as synonyms, and refer to the compounds present in the extract, which exhibit an activity that is similar to at least one of the above-defined activities of the extract.

In yet another embodiment, the present invention relates to a method for obtaining the extract of the plant *Calotropis procera*.

Due to the fact that the extract according to the invention exerts an anti-tumor activity the extract according to the invention is particularly useful for the treatment of diseases such as cancer. Therefore, in another aspect, the present invention relates to pharmaceutical compositions comprising the above-described extract or at least one active compound thereof.

Furthermore, since the extract according to the invention exerts an anti-poisonous activity as well, it is also particularly suitable to be combined with other therapeutic compounds, such as other medicaments, which show toxic side effects. Therefore, in another embodiment, the present invention relates to a pharmaceutical composition comprising the above-described

extract or at least one active compound thereof, and a second compound, which exerts a pharmacological activity having toxic side effects.

Furthermore, the present invention relates to the use of said extract and/or at least one active compound thereof as a medicament. The present invention further relates to the use of the extract or at least one active compound thereof in the preparation of a medicament for the treatment of diseases, in particular cancer.

In another embodiment, the present invention further relates to a method for treating cancer.

Other objects and advantages of the present invention will become apparent from the following detailed description taken in conjunction with the accompanying drawings.

Detailed description of the figures

Figure 1 describes the overall growth of different types of tumors as a function of the concentration of plant extract according to the invention.

Figures 2 to 11 show the *in vitro* effects of the plant extract according to the invention on different types of human cancer cell lines, being breast cancer cell lines in figure 2, sarcoma cancer cell lines in figure 3, pancreatic cancer cell lines in figure 4, melanoma cancer cell lines in figure 5, colon cancer cell lines in figure 6, glioma cancer cell lines in figure 7, lung cancer cell lines in figure 8, bladder cancer cell lines in figure 9, prostate cancer cell lines in figure 10, and head and neck cancer cell lines in figure 11.

Figures 12 to 16 represent the *in vitro* effects of the extract according to the invention on the cell cycle kinetics of cells corresponding to five different human cell lines, HCT-15 (figure 12), RPMI (figure 13), A172 (figure 14), J82 (figure 15) and Hs683 (figure 16). The upper part of the figures shows overall cell growth, as the percentage of living cancer cells to control cells, at different doses of the extract. The middle and lower parts of the figures show the effect on the cell cycle kinetics of the extract at different concentration after 24 hours and 72 hours respectively. Statistical significance was evaluated with the Student's t-test where *: $p<0.05$; **: $p<0.01$: ***: $p<0.001$.

Figures 17 to 20 represent the *in vitro* apoptotic-inducing and necrosis-inducing effects of the extract according to the invention on cells corresponding to four different human cell lines, HCT-15 (figure 17), A172 (figure 18), J82 (figure 19) and Hs683 (figure 20). The upper part of the figures shows apoptotic-inducing and necrosis-inducing effects of the extract at different doses after 24 hours, the lower part of the figures after 72 hours.

Figure 21 represents the effects of the extract on an *in vivo* lymphoma cancer model. The extract was administered by intra-peritoneal injection at doses of 2.5mg/kg; 5mg/kg and 10mg/kg extract in P388 lymphoma-tumor bearing mice. Figure 21A represents the effects of the extract on the weight of control (untreated) mice and in treated mice. Figure 21B represents the effects of the extract on the tumor growth in control mice and in treated mice.

Figure 22 represents the effects of the extract on an *in vivo* lymphoma cancer model. The extract was administered by intra-peritoneal injection at doses of 0.63mg/kg; 1.25mg/kg and 2.5mg/kg extract in P388 lymphoma-tumor bearing mice. Figure 22A represents the effects of the extract on the weight of control mice and treated mice. Figure 22B represents the effects of the extract on the tumor growth in control mice and in treated mice.

Figure 23 represents the effects of the extract on an *in vivo* melanoma cancer model. The extract was administered by intra-peritoneal at doses of 2.5mg/kg; 1.25mg/kg and 0.63mg/kg extract in B16-melanoma bearing mice. Figure 23A represents the effects of the extract on the weight of control (untreated) mice and treated mice. Figure 23B represents the effects of the extract on the tumor growth in control mice and in treated mice.

Figure 24 represents the effects of the extract on an *in vivo* melanoma cancer model. The extract was administered per os at doses of 0.63mg/kg; 1.25mg/kg and 2.5mg/kg extract in B16-melanoma bearing mice. Figure 24A represents the effects of the extract on the weight of control mice and treated mice. Figure 24B represents the effects of the extract on the tumor growth in control mice and in treated mice.

Figure 25 represents the effects of the extract on an *in vivo* breast cancer model. The extract was administered by intra-peritoneal injection at doses of 2.5mg/kg; 5mg/kg and 10 mg/kg extract in MXT-HI breast cancer bearing mice. Figure 25A represents the effects of the

extract on the weight of control (untreated) mice and treated mice. Figure 25B represents the effects of the extract on the tumor growth in control mice and treated mice.

Figure 26 represents the statistical general fit for the MXT-HI breast cancer bearing mice of the test groups (i.e. intra-peritoneal injection of the mice at extract doses of 2.5mg/kg; 5mg/kg and 10 mg/kg) and the control group, with regard to survival (Kaplan-Meier statistical analysis).

Figure 27 represents the effects of the extract according to the invention at doses of 5mg/kg and 1.25 mg/kg on the amount of white blood cell, red blood cell, the hemoglobin and hematocrite concentration at day three after intra-peritoneal injection in mice compared to control mice.

Figure 28 illustrates the anti-poisonous effects of the extract according to the invention when combined with two anti-tumors drugs adriamycine and vincristine.

Detailed description of the invention

Properties of the extract

The *Calotropis procera* plant, belonging to the family of the *Asclepiadaceae*, is a plant growing in Africa and Asia. This plant is used in traditional folk medicine and has also been studied with respect to a considerable number of uses such as an anti-pyretic, anti-malarial, anti-diarroeal, analgesic, anti-inflammtory gastric, mucosal protector and as an insecticidal, anti-tussive, antibacterial, wound healing, muscle relaxant. The stems, flowers and leaves of *Calotropis procera* plant are known to contain certain compounds known as cardenolides. Recently, cardiotoxic activity has been attributed to these cardenolides, and they are exploited in human therapies for treating cardiac insufficiencies.

Unexpectedly, the present invention relates to an extract of the *Calotropis procera* plant showing another type of activity, in particular an "anti-tumor activity". Moreover, it was demonstrated that the extract of the *Calotropis procera* plant shows *in vitro* as well as *in vivo* anti-tumor effects. The extract according to the invention induces a dramatic reduction in cell growth and shows a pro-apoptotic activity. These properties are further illustrated in the examples 3 and 4. In addition, it has been in-vivo shown by the inventors that the extract according to the invention is highly active against several types of cancers. As is shown in the

examples described below, the extract according to the invention, exerts significant anti-tumor effects on several tumor models tested, which represent a broad panel of histological tumor types, including breast cancer, lymphomas, melanomas. These models are clinically relevant because they mimic specific clinical stages of human cancers.

Surprisingly, the extract according to the invention is highly active at relatively low doses. The extract can exert an anti-tumor activity at low doses in the range of 0.4 to 2.2 mg/kg and preferably in the range of 0.5 to 2 mg/kg. The high anti-tumor activity at low doses indicates that the extract is highly active. Surprisingly, as will also become clear when reading the examples given below, the extract according to the invention shows significantly higher anti-tumor effects when assayed at chronically low doses, i.e. 0.6 mg/kg to 1.25 mg/kg, than at high doses, i.e. at doses of 5 mg/kg to 10 mg/kg. The Maximum Tolerated Dose (MTD) index of the extract according to the invention, referring to the maximum amount of the extract, which can be administered acutely to healthy animals, is 20mg/kg extract. This value indicates that the extract according to the invention has a broad therapeutic window.

The terms "toxicity" or 'toxic effects" relate to the detrimental effect(s) a compound may have on healthy cells, tissues or organs. Another important property of the extract according to the invention includes its low toxicity level. It was demonstrated that the extract does not induce *in vivo* hematological changes, does not induce weight loss and shows minor side effects on different types of organs and tissues. In fact, the toxicity level of the extract according to the invention is surprisingly low. The extract according to the invention combines the essential features of a good anti-tumor activity, a low level of toxicity and a minimal induction of detrimental side effects.

Furthermore, the extract of the plant *Calotropis procera* according to the invention also shows an "anti-poisonous activity". As mentioned above, the term "anti-poisonous activity", as used herein, refers to the ability of the extract according to the invention to attenuate or reverse the toxic effects of compounds. Surprisingly, the inventors have demonstrated that combination of the extract according to the invention with other compounds, having toxic effects enables to reduce the toxic effects of these compounds. For instance, example 8 illustrates that the combination of the extract according to the invention with well known anti-cancer drugs, such as vincristine or adriamycine, enables to reduce some toxic effects induced by these anti-cancer drugs.

Composition of the extract

The extract according to the invention comprises one or several active compounds, i.e. compounds present in the extract, which exhibit an activity similar to at least one of the above-defined activities of the extract. The main activities of the extract are an anti-tumor activity and an anti-poisonous activity. As it will be understood by the one skilled in the art, an active compound present in the extract may exerts only one of these properties, or even both of these properties.

In another embodiment, the extract according to the invention comprises at least two of the active compounds selected from the group comprising asclepin, calactin, voruscharin, calotropin, calotropagenin, uzarigenin, calotoxin, uscharin and uscharidin. Unexpectedly, the combination of at least two of these active components, which are also referred to as cardenolide compounds, provides a synergistic effect to the anti-tumor activity of the extract.

As it will be understood, the extract according to the invention may further contain one or more active compounds, which do not belong to the group of the cardenolides, and other compounds. In another embodiment, the extract contains at least one of the compounds which are represented in Table 1.

TABLE 1 Some of the compounds contained in the extract according to the invention

Compounds present in the extract according to the invention	3-O-ACETYL-CALOTROPIN, ALPHA-AMYRIN, ALPHA-AMYRIN-BENZOATE, ALPHA-CALOTROPEOL, ALPHA-LACTUCEROL, ALPHA-LACTUCERYL-ACETATE, ALPHA-LACTUCERYL-ISOVALERATE, ARABINOSE, ASH, BENZOYLISOLINEOLONE, BENZOYLLINEOLONE, BETA-AMYRIN, BETA-AMYRIN-BENZOATE, BETA-CALOTROPEOL, BETA-SITOSTEROL, CAOUTCHOUC, COROGLAUCOGENIN; COROTOXIGENIN; D-GLUCOSAMINE, FRUGOSIDE, GIGANTEOL, GIGANTIN, GLUCOSE, HISTAMINE, ISOGIGANTEOL, ISOLACTUCEROL, ISOLINEOLONE, LAURANE LINEOLONE, LINOLEIC-ACID, LINOLENIC-ACID, MELISSYL-ALCOHOL, MUDARINE, OLEIC-ACID, PALMITIC-ACID, PROCEROSIDE, PSEUDOCALOTROPAGENIN, RHAMNOSE STIGMASTEROL, SYRIOGENIN TARAXASTEROL, TARAXASTEROL-BENZOATE, TRYPSIN

In another embodiment, the present invention relates to an active compound isolated from the extract according to the invention.

Method of extraction

According to another embodiment, the extract according to the invention can be obtained by an alcoholic extraction, in particular by a methanol extraction.

In another embodiment, the present invention relates to a method for obtaining the extract according to the invention comprising the steps of:

a) extracting the starting material of said *Calotropis procera* plant, said starting material being selected among fruits, aerial parts, subterranean parts, and their mixtures, in an aliphatic alcohol, by dissolving the starting material in said alcohol thereby obtaining a suspension of said material in said alcohol, stirring said suspension; and filtering said suspension by fritted glass thereby obtaining a first filtrate and a first solid part;
b) extracting said first solid part in an aliphatic alcohol thereby obtaining a second filtrate and a second solid part;
c) combining said first and said second filtrate thereby obtaining a combined filtrate; and
d) evaporating said combined filtrate under vacuum thereby obtaining an oily residue.

In a preferred embodiment, the starting material of said *Calotropis procera* plant is selected from subterranean parts, in particular from roots.

In another embodiment, the aliphatic alcohol used to extract the starting material according to the invention is methanol. In yet another embodiment, the residue obtained in the extraction process is taken up in a solvent, in particular in a pharmaceutically acceptable solvent.

Applicability of the extract

Due to its interesting properties; in particular its anti-tumor activity, its anti-poisonous activity, its low level of toxicity and its high activity at low doses, the extract according to the invention or active compounds thereof are particularly suitable for use as a medicament for the treatment of diseases, and in particular for treating cancer.

In another embodiment, the invention relates to the use of an extract according to the invention, or an active compound thereof, as a medicament.

In yet another embodiment, the invention also relates to the use of an extract according to any the invention, or an active compound thereof, for the preparation of a medicament in the

treatment of cancer. In particular the extract according to the invention, or an active compound thereof is used for the preparation of a medicament in the treatment of a cancer selected from the group comprising breast cancer, lymphoma, sarcoma, pancreatic cancer, melanoma, colorectal cancer, glioma, lung cancer, bladder cancer, head and neck cancer, prostate cancer, liver cancer and hematological cancer.

As mentioned above, the extract according to the invention also comprises an anti-poisonous activity. The inventors have demonstrated that a combination of the extract according to the invention or active compounds thereof with a second compound having a toxic activity enables to reduce the toxic activity of this second compound. Therefore, the *Calotropis procera* extract according to the invention may also be combined in a medicament with other drugs, in particular with other anti-cancer drugs.

Pharmaceutical compositions comprising the extract

In another embodiment, the present invention relates to a pharmaceutical composition for the treatment of cancer comprising a therapeutic effective amount of the extract of *Calotropis procera* according to the invention or an active compound thereof, and a pharmaceutical acceptable carrier.

The term "therapeutically effective amount" as used herein means that amount of extract or active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue, system, animal or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the disease being treated.

The pharmaceutical composition can be prepared in a manner known to one of skill in the art. For this purpose, the extract according to the invention and/or any active compound thereof, one or more solid or liquid pharmaceutical carriers and, if desired, in combination with other pharmaceutical active compounds, are brought into a suitable administration form or dosage form which can then be used as a pharmaceutical in human medicine or veterinary medicine.

Particular forms of the pharmaceutical composition may be, for example, solutions, suspensions, emulsions, creams, tablets, capsules, nasal sprays, lipsomes or micro-reservoirs, especially compositions in orally ingestible or sterile injectable form, for example,

as sterile injectable aqueous or oleaginous suspensions or suppositories. The preferred form of composition contemplated is the dry solid form, which includes capsules, granules, tablets, pills, boluses and powders. The solid carrier may comprise one or more carriers, e.g. lactose, fillers, disintegrating agents, binders, e.g. cellulose, carboxymethylcellulose or starch or anti-stick agents, e.g. magnesium stearate, to prevent tablets from adhering to tabletting equipment. Tablets, pills and boluses may be formed so as to disintegrate rapidly or to provide slow release of the active ingredient.

Furthermore, due to its anti-poisonous activity, the extract according to the invention or active compounds thereof, are also particularly suitable to be combined with other therapeutic compounds which exert a pharmacological activity having toxic side effects, such as other medicaments, which show toxic side effects.

The "therapeutic compound, which exerts a pharmacological activity having toxic side effects" may include any compound that is used for the treatment of any disease but which induces unwanted toxic effects. Preferably, such compounds are compounds that are used in the treatment of cancer. For instance, the extract or active compounds thereof can be combined with one or more than one other compound that has an anti-tumor effect. Since two or more compounds having an anti-tumor effect are combined, an improved anti-tumor activity can be obtained. In addition, such combinations also enable to reduce the toxic side effects, which are induced by one or several of the anti-tumor compounds.

A combination of the extract according to the invention or active compounds thereof with another therapeutic compound may involve a separate use of the extract or active compound thereof and the other therapeutic compound. In particular, the combination may involve the use of the extract according to the invention or active compounds thereof prior (pre-treatment) to or after (post-treatment) the use of the other therapeutic compound.

Alternatively, a combination of the extract or active compounds thereof with another therapeutic compound may also involve a mixture of both elements. Therefore, in a preferred embodiment, the present invention relates to a pharmaceutical composition comprising a first component, said first component being the above-described extract or at least one active compound thereof, and a second component, said second component comprising a therapeutic compound, which exerts a pharmacological activity having toxic side effects.

Method of treatment

Due to the favorable anti-tumor properties of the extract according to the present invention, said extract is particularly useful in the treatment of individuals suffering from cancer. In another embodiment, the present invention also relates to a method of treatment of cancer comprising administering to an individual in need of such treatment a pharmaceutical composition according to the invention. Due to its low level of toxicity and its minimal side effects, use of the extract in a pharmaceutical composition for the treatment of cancer will involve minimal side effects. As a consequence, the extract according to the invention may be used during a longer period of time during the treatment of cancer.

In particular, in a preferred embodiment, the invention relates to a method for treating cancer, whereln the cancer is selected from the group comprising breast cancer, lymphoma, sarcoma, pancreatic cancer, melanoma, colorectal cancer, glioma, lung cancer, bladder cancer, head and neck cancer, prostate cancer, liver cancer and hematological cancer.

For these purposes, the pharmaceutical composition of the present invention may be administered orally, parenterally, i.e. including subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques, by inhalation spray, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles.

In accordance with the method of the present invention, said pharmaceutical composition can be administered separately at different times during the course of therapy or concurrently in divided or single combination forms. The present invention is therefore to be understood as embracing all such regimes of simultaneous or alternating treatment and the term "administering" is to be interpreted accordingly.

Essentially, the primary modes of treatment of solid tumor cancers comprise surgery, radiation therapy and chemotherapy, separately and in combination. The extract according to the invention are suitable for use in combination with these medicinal techniques. The extract according to the invention may be useful in increasing the sensitivity of tumor cells to radiation in radiotherapy and also in potentiating or enhancing damage to tumors by chemotherapeutic agents. The extract according to the invention may also be useful for

sensitizing multidrug-resistant tumor cells. The compounds according to the invention are useful therapeutic compounds for administration in conjunction with other DNA-damaging cytotoxic drugs or radiation used in radiotherapy to potentiate their effect.

It will be understood, that the pharmaceutical composition according to the invention can be administered to humans in specific dose levels and at specific frequency of dosage which may be varied for any particular patient and which will depend upon a variety of factors including the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition of the patient undergoing therapy.

The following examples are meant to illustrate the present invention. These examples are not to be considered as limiting the scope of the invention. A first example illustrates the extraction of an extract according to the invention form the plant *Calotropis procera*. Examples 2 to 4 relate to the *in vitro* anti-tumor characterization of the extract according to the invention. Examples 5 to 7 describe the *in vivo* anti-tumor characterization of the extract according to the invention. Example 8 illustrates the anti-poisonous effects of the extract according to the invention.

Examples

Example 1 Extraction process of the extract according to the invention from the plant Calotropis procera

The extract according to the invention was isolated form the roots of *Calotropis procera* plant (*Asclepiadiaceae* family) by methanol. About 10 grams of plant was put in an erlenmeyer with 150 ml of methanol. The suspension was shaken magnetically for 12 hours and then filtrated on glass frit. The remaining solid was extracted a second time by methanol for 2 hours. Both filtrates were combined and evaporated under vacuum by using rotavapor. The residue constituted the methanolic extract.

Analysis of the methanolic extract revealed the presence of several types of compounds. A particular group of compounds identified in the extract comprises cardenolides such as asclepin, calactin, vorusharin, calotropin, calotropagenin, uzarigenin, usharin and usharidin. In addition, some other compounds, present in the extract comprise, but are not limited to, 3-O-ACETYL-CALOTROPIN, ALPHA-AMYRIN, ALPHA-AMYRIN-BENZOATE, ALPHA-

CALOTROPEOL, ALPHA-LACTUCEROL, ALPHA-LACTUCERYL-ACETATE, ALPHA-LACTUCERYL-ISOVALERATE, ARABINOSE, BENZOYLISOLINEOLONE, BENZOYLLINEOLONE, BETA-AMYRIN, BETA-AMYRIN-BENZOATE, BETA-CALOTROPEOL, BETA-SITOSTEROL, CAOUTCHOUC, COROGLAUCOGENIN; COROTOXIGENIN; D-GLUCOSAMINE, FRUGOSIDE, GIGANTEOL, GIGANTIN, GLUCOSE, HISTAMINE, ISOGIGANTEOL, ISOLACTUCEROL, ISOLINEOLONE, LAURANE LINEOLONE, LINOLEIC-ACID, LINOLENIC-ACID, MELISSYL-ALCOHOL, MUDARINE, OLEIC-ACID, PALMITIC-ACID, PROCEROSIDE, PSEUDOCALOTROPAGENIN, RHAMNOSE STIGMASTEROL, SYRIOGENIN TARAXASTEROL, TARAXASTEROL-BENZOATE and TRYPSIN.

Example 2 Effect of the extract according to the invention on overall cell growth of a cell line

This example illustrates the anti-tumor activities of the extract according to the invention on different types of cancer.

In order to characterize the *in vitro* activities of the extract according to the invention, MTT tests were carried out. The MTT test, which is a well known test in the art, is an indirect technique that rapidly measures, i.e. within 5 days, the effect of a given product on the overall growth of a cell line. This test measures the number of metabolically active living cells that are able to transform the MTT product (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide), having a yellowish color, to the blue product formazan by mitochondrial reduction only performed by living cells. The amount of formazan obtained at the end of the experiment is measured with a spectrophotometer and is directly proportional to the number of living cells.

Forty-eight human cancer cell lines, described in Table 2, were tested in the presence of the extract according to the invention. These cell lines covered ten histological types, being pancreatic cancer, sarcoma, breast cancer, melanoma, colon cancer, glioma, lung cancer, bladder cancer, prostate cancer and head and neck cancer. The cells were allowed to grow in flat bottomed 96-well micro-wells with 100 µl of cell suspension per well and between 3,000 and 5,000 cells/well depending on cell type. Each cell line was seeded in its own cell culture medium as indicated in Table 2.

TABLE 2 Human cancer cell lines and corresponding cell culture medium used for the MTT experiments

Cell lines	ATCC code	Tissue	Medium
BxPC-3	CRL-1687	Pancreatic	RPMI 10% serum
MiaPACA-2	CRL-1420	Pancreatic	DMEM glucose 10%
PANC-1	CRL-1469	Pancreatic	OPTIMEM 5%
CAPAN-1	HTB-79	Pancreatic	RPMI 10% serum
CFPAC-1	CRL-1918	Pancreatic	Iscove's 10% serum
Hs766T	HTB-134	Pancreatic	OPTIMEM 5%
SU.86.86	CRL-1837	Pancreatic	RPMI 10% serum
SK-LMS-1	HTB-88	Sarcoma	MEM 10% serum
SK-UT-1B	HTB-115	Sarcoma	MEM 10% serum AA
HT-1080	CCL-121	Sarcoma	MEM 5% serum
Hs729	HTB-153	Sarcoma	MEM 10% serum AA
MES-SA	CRL-1976	Sarcoma	MEM 5% serum
RD	CCL-136	Sarcoma	MEM 5% serum
A204	HTB-82	Sarcoma	MEM 5% serum
MCF-7	HTB-22	Breast	MEM 5% serum
T-47D	HTB-133	Breast	MEM 5% serum
MDA-MB-231	HTB-26	Breast	DMEM Nut mix 10% serum
ZR-75-1	CRL-1500	Breast	MEM 5% serum
Hs578T	HTB-126	Breast	MEM 5% serum
SK-MEL-28	HTB-72	Melanoma	RPMI 10% serum
HT-144	HTB-63	Melanoma	MEM 10% serum AA
C-32	CRL-1585	Melanoma	MEM 5% serum
Malme-3M	HTB-64	Melanoma	MEM 5% serum
G-361	CRL-1424	Melanoma	OPTIMEM 5% serum
HCT-15	CCL-225	Colon	MEM 5% serum
LoVo	CCL-229	Colon	MEM 5% serum
DLD-1	CCL-221	Colon	MEM 10% serum AA
Ls-174T	CL-188	Colon	MEM 10% serum AA
HT29	HTB-38	Colon	MEM 10% serum AA
WiDR	CCL-218	Colon	MEM 10% serum AA
SW948	CCL-237	Colon	OPTMEM 5 % serum
A172	CRL-1620	Glioma	MEM 5% serum
H4	HTB-148	Glioma	MEM 5% serum
Hs683	HTB-138	Glioma	MEM 5% serum
SW1088	HTB-12	Glioma	MEM 5% serum
U-118 MG	HTB-15	Glioma	MEM 5% serum
U-87 MG	HTB-14	Glioma	MEM 5% serum
U-373 MG	HTB-17	Glioma	MEM 5% serum
A427	HTB-53	Lung	MEM 5% serum
A549	CCL-185	Lung	MEM 5% serum
J82	HTB-1	Bladder	MEM 5% serum
T24	HTB-4	Bladder	MEM 5% serum
PC3	CRL-1435	Prostate	MEM 5% serum

Detroit	CCL-138	Head and neck	MEM 10% serum AA
RPMI	CCL-30	Head and neck	MEM 10% serum AA
FaDu	HTB-43	Head and neck	MEM 10% serum AA
SCC9	CRL-1629	Head and neck	MEM 10% serum AA
SCC25	CRL-1628	Head and neck	MEM 10% serum AA

Where AA means "Amino Acids"

After a 24-hour incubation period at 37°C the culture medium was replaced by 100 µl of fresh medium in which the extract according to the invention was dissolved at the different concentrations of 0.01 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml. Each experimental condition was carried out in sextuplicate.

After 72 hours of incubation at 37°C with the drug, i.e. experimental conditions or without the drug, i.e. control, the medium was replaced by 100 µl MTT at the concentration of 1 mg/ml dissolved in RPMI. The micro-wells were then incubated for 3 hours at 37° C and centrifuged at 400g for 10 minutes. The MTT was removed and the formed formazan crystals were dissolved in 100 µl DMSO. The micro-wells were shaken for 5 minutes and read on a spectrophotometer at 2 wavelengths at 570 nm corresponding to the maximum formazan absorbance wavelength, and at 630 nm, which is the background noise wavelength.

For each experimental condition, the mean OD associated with the standard error of the mean (SEM) for each condition, i.e. 6 wells, was calculated. The percentage of remaining living cells was calculated in comparison with control. Results of these experiments are represented in figures 1 to 11.

Figure 1 represents the overall results for the ten histological types. As indicated the extract according to the invention exerted an anti-tumor effect on all histological types tested. The human tumor cell lines issued from bladder presented a sensitivity to the extract according to the invention which was weaker than the remaining nine histological types. The concentration at which the plant extract killed 50% of the cells population, the so-called IC_{50} value, was determined. Said IC_{50} value comprised between 0.5 µg/ml and 1 µg/ml for the cell lines issued from the breast and the pancreas cell lines. A more important anti-tumor effect with a IC_{50} value of 0.5-0.1 µg/ml was obtained for sarcoma, melanoma, glioma, lung, head and neck and colorectal, cancer cell lines. The PC3 prostate cancer cells exhibited a marked sensitivity, with an IC_{50} value in range of 0.01 to 0.05 µg/ml.

As illustrated on figure 1, the mean IC_{50} value of the 5 breast cancer cell lines ranged between 0.5 and 1 µg/ml. The overall growth of the 5 individual breast cancer cell lines, T-47D, MDA-MB-231, ZR-75-1, MCF-7 and Hs578T, is further illustrated in figure 2. The cell lines T-47D, MDA-MB-231, ZR-75-1 and Hs578T exhibited similar sensitivities to the extract according to the invention, while MCF-7 cells had their overall growth reduced more rapidly than the remaining four breast cancer models.

As illustrated on figure 1, the mean IC_{50} value of the 7 sarcoma cancer cell lines (see Table 1) ranged between 0.5 and 0.1 µg/ml. The overall growth of the individual cell lines is further illustrated in figure 3. The extract according to the invention induced the most important anti-tumor effect on the A204 cell line. In fact, the IC_{50} value appeared to be between 0.1 and 0.05 µg/ml. The Hs729 cell line was considered to the least sensitive of the 7 sarcoma cell lines. It nevertheless showed IC_{50} values for extract according to the invention ranging between 1 and 5 µg/ml.

As illustrated on figure 1, the mean IC_{50} value of the 7 pancreatic cancer cell lines (see Table 1) ranged between 0.5 and 0.1 µg/ml. Similar overall growths were observed for 6 of the 7 pancreatic cancer cell lines (Figure 4). Of these 7 cell lines, Panc-1 seemed to be the most sensitive to the extract according to the invention and Hs766T the least. The IC_{50} values of these 2 lines ranged between 0.1 and 0.5 µg/ml, and between 1 and 5 µg/ml respectively.

As illustrated on figure 1, the mean IC_{50} value of the 5 melanoma cancer cell lines (see Table 1) ranged between 0.5 and 0.1 µg/ml. The extract according to the invention reduced the overall growth of all the melanoma cell lines by more than 60% at concentrations equal to or higher than 5 µg/ml (Figure 5). Malme-3M and G-361 were the most sensitive of the 5 melanoma cell lines. They exhibited a similar overall growth, with the IC_{50} value ranging between 0.1 and 0.5 µg/ml.

As illustrated on figure 1, the mean IC_{50} value of the 7 colon cancer cell lines (see Table 1) ranged between 0.5 and 0.1 µg/ml. Of the different colorectal cancer cell lines tested (Figure 6) the LoVo line had an IC_{50} value of around 0.1 µg/ml and was considered to be the most sensitive colorectal line. The SW948 tumor cell line was considered to be the least sensitive, with a IC_{50} value ranging between 0.5 and 1 µg/ml.

As illustrated on figure 1, the mean IC_{50} value of the 7 human glioma cancer cell lines (see Table 1) ranged between 0.5 and 0.1 µg/ml. Hs683 was the most sensitive cell line to extract (Figure 7). Its IC_{50} value was near 0.05 µg/ml. In contrast, the growth of A172 cells was not affected by the extract according to the invention at concentrations from 0.01 to 10 µg/ml. The IC_{50} value ranged between 10 and 50 µg/ml.

As illustrated on figure 1, the mean IC_{50} value of the 2 human non-small-cell-lung cancer cell lines (NSCLC) ranged between 0.1 and 0.5 µg/ml. Figure 8 shows that both lines were sensitive to the extract according to the invention.

As illustrated on figure 1, the mean IC_{50} value of the 2 human bladder cancer cell lines (see Table 1) ranged between 50 and 100 µg/ml. The 2 lines exhibited marked differences in term of sensitivity to the extract according to the invention (Figure 9). In fact, at 5 µg/ml of the extract according to the invention reduced the overall growth of the T24 cell line by more than 80% while the overall growth of the J82 cell line was only decreased by 32% at 100 µg/ml. The J82 cell line seemed very weakly sensitive to extract according to the invention.

The overall growth of 1 prostate cancer cell line (the PC3 cell line) was evaluated by MTT assay when the cells had been treated with the extract according to the invention. The extract induced an inhibition of overall growth of about 90% at between 100 µg/ml and 0.5 µg/ml. The IC_{50} value was around 0.1 µg/ml (Figure 10).

As illustrated on figure 1, the mean IC_{50} value of the 5 head and neck cancer cell lines ranged between 0.1 and 0.5 µg/ml. Similar overall growths were observed for 4 of the 5 head and Neck cancer cell lines (Figure 11). Of these 5 cell lines, SCC9 seemed to be the least sensitive. It nevertheless showed IC_{50} values for the extract of around 5 µg/ml.

Summarized, the extract according to the invention exert a dramatic anti-tumor effect on forty-six of the forty-eight human cancer cell lines assayed in the experiments described above. These anti-tumor effects correspond to marked decreases in the overall growth of these human cancer models representing a very broad panel of histological types.

Example 3 Effect of the extract according to the invention on cell kinetics

This example illustrates the cytostatic effect of the extract according to the invention. According to the experiments performed by means of the MTT colorimetric assay described in example 2, it is clear that the extract according to the invention dramatically decreases the overall growth of most of the fourty-eight human cancer cell lines submitted to the MTT assay. In the following examples it was investigated whether this extract-induced decrease in overall growth corresponds either to modifications occurring at the levels of the cell cycle kinetics (example 3), or the induction of apoptosis (see example 4), or of both.

The cell cycle is in general divided in several phases comprising a G0/G1, S and G2/M phase. Modifications taking place around the proteins controlling cell proliferation and/or cell death may constitute one target of the active compounds, present in the extract according to the invention. Modifications to cell cycle kinetics can be investigated by means of flow cytometry using different fluorophores f.e. propidium iodide, orange acridine and ethidium bromide etc. Flow cytometry enables each cancer cell (running to several thousands) to be located into the cell cycle. Changes in the DNA histogram pattern are thus used to characterize the mechanism of action of various cytotoxic drugs. The data resulting from the flow cytometry analysis is processed graphically or mathematically in order to derive meaningful estimates of the G1, the S and the G2/M compartments. Most mathematical processing is based on certain models and assumptions.

Cell lines were seeded in flasks (25 cm^2 area) containing 7 ml of culture medium. After 48 hours incubation at 37°C the cell culture medium was replaced by fresh medium in which the extract according to the invention had been dissolved at concentrations which kill 50%, (IC_{50}), 30% (=IC_{30}) and 10% (IC_{10}) of the cell population. After 24 or 72 hours of treatment the cells were harvested in suspension, washed in Phosphate Buffer Saline (PBS) at 4°C and permeabilized with 70 % ethanol (at 4°C) overnight at –20°C. The cells were then washed with PBS and incubated with propidium iodide solution (80 µg/ml) for 30 minutes at 37°C and afterwards kept at 4°C overnight. Ribonuclease A (3% V/V) was added to induce a double-stranded DNA break. The portrait of the cell cycle was established for each sample. A specific software program incorporated into the flow cytometer was used to define precisely the percentage of cells in the different cell cycle phases. Each cell cycle phase was reported in terms of peak surface and calculated as a percentage. The surface of the entire cell cycle was 100 %. Each experiment was carried out 3 times. The mean percentage of each different

phase and the standard error of the related mean was calculated. Each cell cycle phase of a given condition was compared with the same cell cycle phase of control cells, i.e non-treated cells.

The extract according to the invention is highly effective against human tumor cell lines. The concentrations used in our flow cytometry experiments were chosen in accordance with the MTT results (example 2). The five human cancer cell lines Hs683 (glioma), J82 (bladder) A172 (glioma), RPMI (head-and-neck) and HCT-15 (colon) were tested and doses that killed 10, 30 and 50 percent of the cells were determined (Table 3) in order to investigate whether the extract promoted an accumulation in one of the cell cycle phases when the cultures were treated for 24 or 72 hours with increasing concentrations of the extract.

TABLE 3 Doses of the extract according to the invention killing 10, 30 and 50 % of the cells in five cell lines

Cell lines	IC_{50}	IC_{30}	IC_{10}
HCT-15	0.25 µg/ml	0.1 µg/ml	0.05 µg/ml
RPMI	0.25 µg/ml	0.1 µg/ml	0.05 µg/ml
A172	25 µg/ml	—	10 µg/ml
J82	—	50 µg/ml	10 µg/ml
Hs683	0.05 µg/ml	—	0.025 µg/ml

The HCT-15 cell cycle analyses (Figure 12) showed that the distribution of the cell population was similar to the control after 24 hours of treatment independent of the concentrations tested. On the other hand, a prominent S-population appeared in the cells treated with 0.25 µg/ml extract for 72 hours. The S fraction represented 21% of the cells in control and reached 59% upon treatment with the extract at 0.25 µg/ml. The increase in the S fraction was accompanied by a loss of cells in the G0/G1 phase. The G0/G1 population underwent a marked decrease from 71% to 29%.

The analyses of the RPMI cell cycle (Figure 13) showed that the distribution of the cell population treated with the said extract at 0,05 µg/ml was similar to the control after 24 hours of treatment. A weak increase in phase S was observed at 0,1 and 0,25 µg/ml. In contrast, an large increase in the S-population occurred when the RPMI cell line was incubated for 72 hours with the extract at the three concentrations tested. There were about 71%, 69% and 62% of cells in the S-phase when the extract was assayed at 0.05 µg/ml, 0.1 µg/ml and 0.25

µg/ml, respectively. Concomitantly, the percentage of cells in the G0/G1 phase decreased markedly and the G2/M phase increased slightly, reaching 29% of the cell population.

The A172 cell line was treated with the extract at 10 and 25 µg/ml, which corresponds to the IC_{10} and IC_{50}. The results (Figure 14) showed that whatever the concentrations tested the extract induced an increase in the G0/G1 phase after 24 hours of treatment. Thirty-nine percent of the cells were in the G0/G1 phase in control while the cells treated with the extract reached 60%. Concomitantly with the accumulation in the G0/G1 phase, we observed a slight accumulation in the G2/M phase at the greatest concentration tested. The effect observed after 24 hours of treatment disappeared after 72 hours. Indeed, no significant change was observed in the different cell cycle phases.

The J82 cell line is hardly sensitive to the extract (cfr. MTT assay). The concentrations chosen for flow cytometry corresponded to about IC_{10} and IC_{30} (Figure 15). After 24 hours of treatment a slight accumulation in G0/G1 phase was obtained whatever the concentration tested. On the other hand, 30 and 47% respectively of the cell population was in the S phase, when the cell were treated for 72 hours with the extract at 50 and 100 µg/ml while the control condition reached only 18%. At 100 µg/ml, an accumulation in G2/M phase could also be observed.

After 24 hours of treatment with the extract at 0.05 µg/ml the Hs683 cells had accumulated in the G0/G1 phase and attained 70% as compared to control (58%) (Figure 16). We observed concomitantly a decrease in the percentage of cells in the S phase. In the same way, at the both concentrations most of the cells were in the G0/G1 phase after 72 hours of treatment. Indeed, more than 60% of the cells were in this phase while the cells in this phase in control represented 46% of cell population.

In conclusion, the extract according to the invention principally induces an accumulation in the G0/G1 phase and under such circumstances this indicates that the extract has a cytostatic effect.

Example 4 Pro-apoptotic effect of the extract according to the invention

This example illustrates the pro-apoptotic effect of the extract according to the invention.

Cell death may occur in two different ways, either accidentally or as genetically programmed. Accidental cell death, also referred to as necrosis essentially occurs as the results of e.g. physical or biological aggression. Apoptosis refers to the form of cell death which is genetically programmed cell death and which occurs under normal physiological conditions. After the induction of apoptosis, a cascade of events is induced in the cell, which comprises the activation of cell death receptors, the activation of a serie of cytosolic proteases, the formation of apoptotic bodies, the fragmentation of the DNA.

The effect of the extract according to the invention on the apoptosis pathway was investigated on the four human cancer cell lines: Hs683 (glioma), J82 (bladder), A172 (glioma) and HCT-15 (colon).

Cells were seeded in flasks (25 cm^2 area) containing 7 ml of culture medium. After a 48-hours incubation at 37°C the cell culture medium was replaced by fresh medium in which the extract according to the invention was dissolved at different concentrations that killed 50% and 30% of cell population. After 24 or 72 hours of treatment cells were harvested in suspension and counted. 250.000 cells were centrifuged for 10 minutes at 1700 rpm at 4°C. The pellet was washed with PBS at 4°C and incubated with annexin V-FITC and propidium iodide solution for 15 minutes at 4°C in the dark. For each sample, data from approximately 10,000 cells were recorded on logarithmic scale. Software incorporated into the flow cytometer was used to define precisely the percentage of cells in apoptotic and/or necrotic pathway, and the normal cells. Each experiment was realized two times. The mean of percentage of both the apoptotic and the necrotic way and the standard error of the related mean was calculated. Each condition was compared to the control, being non-treated cells.

Four-fold increase of apoptotic HCT-15 cells was observed when cells were treated by the extract at 0.25 µg/ml for 24 hours as compared to the control cells (Figure 17). The A172 cell line seemed to be engaged in an apoptosis and necrosis pathway when the cells were treated for 24 hours by the extract at 25 µg/ml (Figure 18). This tendency was maintained after 72 hours of treatment. The treatment by the extract according to the invention induced the apoptosis of J82 cells in a concentration-dependent manner, which is particularly clear 72 hours after treatment (Figure 19). Also an increase in the percentage of necrotic cells is observed at both concentrations at 72 hours. A 2.5 fold increase in the percentage of Hs683 apoptotic cells was obtained after 24 hours of treatment with the extract at 0.025 µg/ml (Figure 20). After 72 hours of treatment the percentage of apoptotic cells increased in concentration-dependent manner.

In conclusion, the extract according to the invention principally induces an increase in the percentage of apoptotic cells. An effect on the necrosis pathway was obtained under certain conditions.

Example 5 Maximum tolerated dose of the extract according to the invention

In the present example, the MTD index was determined for the extract according to the invention. The Maximum Tolerated Dose (MTD) of a given drug is defined as the maximum amount of a drug which can be administered acutely, i.e. in one intraperitoneal, intravenous, subcutaneous or *per os* single dose, to healthy animals, i.e. animals not grafted with tumors.

The experimental conditions to determine the MTD index of the plant extract according to the invention were the following. The survival times of mice, which are not grafted with a tumor, were recorded up to 14 days post-injection. Six different doses of extract according to the invention were used for the determination of the MTD index. The highest dose administered to tumor-bearing mice was 160 mg/kg. Other doses comprised 5mg/kg, 10mg/kg, 20mg/kg, 40mg/kg and 80mg/kg. Each experimental group was composed of two mice for the determination of the MTD index. Table 4 below shows the data obtained for the MTD index using the extract according to the invention.

TABLE 4 MTD index determination for the extract according to the invention

Administered doses of extract (mg/kg)	Day 1 post administration	Day 14 post administration
1 x 5	--	--
1 x 10	--	--
1 x 20	--	--
1 x 40	X	
1 x 80	XX	
1 x 160	XX	

where x means one dead mouse and -- means all the animals remained alive

According to the definition given above, the MTD index for the extract is 20 mg/kg for single administration in mice. Thus, the Maximum Tolerated Dose (MTD) index of the extract according to the invention, referring to the maximum amount of the extract, which can be

administered acutely to healthy animals, is 20mg/kg extract. This value indicates that the extract according to the invention has a broad therapeutic window.

Example 6 In vivo effects of the extract according to the invention evaluated on three cancer models

This example illustrates the *in vivo* effects of the extract according to the invention on three different cancer models.

The *in vivo* effects of the plant extract according to the invention were studied on mice grafted with different types of tumors, including a lymphoma cancer, a melanoma cancer and a breast cancer. The *in vivo* effects were evaluated with three types of parameters:

- the *cumulative toxicity,* which is evaluated by recording the weights of the tumor-bearing mice during treatment
- the *actual anti-tumor effect* exerted at tumor growth level, which is evaluated by measuring tumor size three times a week by means of a caliper. The actual tumor growth is expressed as an area (mm^2) by multiplying the two largest perpendicular diameters.
- the *survival gain* for the mice treated, which is calculated by means of the T/C index. This index is the ratio between the median survival time of the group of treated mice (T) and that of the control group (C). The extract is considered to be active if the T/C value is above 130 % ($P<0.05$), very active for a value higher than 150% ($P<0.01$) and toxic for a value lower than 70%.

In vivo effects of the extract according to the invention on a lymphoma cancer model

The extract according to the invention was evaluated on the aggressive P388 lymphoma cancer model. In a first experiment three doses, 10mg/kg, 5mg/kg and 2.5mg/kg were compared to control. The mice were inoculated subcutaneously with 10^6 P388 cells at day D0 and treated nine times during the three following weeks at days D5, D7, D9, D12, D14, D16, D19, D21 and D23 post-graft. Each experimental group contained nine mice.

The data detailed in Table 5 show that the mice died before giving them the nine injections of the extract. The number of injections when reaching the T/C index was 7 for the dose of 10mg/kg, 6 for the dose of 5mg/kg and 8 for the dose of 2.5mg/kg.

TABLE 5 Number of dead mice upon administration of the extract according to the invention under the given conditions at different days after grafting

	doses	Number of injections at T/C	Days after grafting								
			14	15	16	19	21	22	23	26	28
EXTRACT	(3x3) 10mg/kg	7	1	1		2	1	1	2	1	
	(3x3) 5 mg/kg	6			2	3			1	2	1
	(3x3) 2.5 mg/kg	8		1	1	2			2	1	2
CONTROL	3x3	6				5	2		2		

Figures 21A illustrates that the extract did not influence the body weight of the mice. Figure 21B shows that the extract significantly induced a decrease in P388 lymphoma growth. The T/C index values for the 7x10 mg/kg administration schedule gave a T/C index of 111 %, the T/C index values for the 6 x 5 mg/kg schedule gave a T/C index of 100 % and the T/C index values for the 8 X 2.5 mg/kg schedule gave a T/C index of 121 %. In conclusion, these results indicated that administration of the extract according to the invention decreased the growth of the P388 lymphoma cancer but did not significantly prolong the survival of the P388 lymphoma-bearing mice at the tested concentrations.

In a second set of experiments, the number of administrations was increased from nine to sixteen, accompanied by a concomitant decrease in the dose per single administration. The subcutaneously-administered doses were 2.5mg/kg, 1.25 mg/kg and 0.63mg/kg. The extract was administered daily, five days a week, for five consecutive weeks (5 x 5 = 25).

The data detailed in Table 6 shows that the mice died before giving them the 25 injections of the extract. The number of injections when reaching the T/C index was 15 for the dose of 2.5mg/kg, 15 for the dose of 1.25mg/kg and 13 for the dose of 0.63mg/kg.

TABLE 6 Number of dead mice upon administration of the extract according to the invention under the given conditions at different days after grafting

	doses	Number of injections at T/C	Days after grafting									
			15	16	19	20	21	22	23	26	28	30
EXTRACT	(5x5) 2.5mg/kg	15	2		1			1		1	3	1
	(5x5) 1.25mg/kg	15	1	1	1					4	1	1
	(5x5) 0.63mg/kg	13		1		2	1	1	1	2	1	
CONTROL	5x5	10			6			1	1	1		

Figure 22B shows that these active extract doses of 0.63 mg/kg and 1.25 mg/kg had negligible toxic effects since the P388 lymphoma-bearing mice lost no weight during treatment. Figure 22A shows that the extract according to the invention markedly decreased P388 lymphoma growth at doses of both 0.63 mg/kg and 1.25 mg/kg. In addition, the T/C index values for the 15 x 2.5 mg/kg administration schedule gave a T/C index of 137 %, that the T/C index values for the 15 x 1.25 mg/kg schedule gave a T/C index of 137 % and that the T/C index values for the 13 X 0.63mg/kg schedule gave a T/C index of 116%. Thus, the 15 administrations of 2.5 mg/kg extract and the 15 administrations of 1.25 mg/kg extract significantly increased the survival of these P388 lymphoma-bearing mice by 37%. Consequently, the treatments at doses of 2.5 mg/kg and 1.25 mg/kg with the extract according to the invention prolonged the survival of P388 lymphoma-bearing mice.

In conclusion, the extract according to the invention exerts significant anti-tumor effects on the aggressive P388 lymphoma cancer model without any loss of body weight in the animals concerned. Also, unexpectedly, the plant extract according to the invention exerts higher anti-tumor activity when assayed chronically at low doses, i.e. around 1.25 to 0.63mg/kg, than at high doses, i.e. around 10mg/kg to 5 mg/kg.

In vivo effects of the extract according to the invention on a melanoma cancer model

The extract according to the invention was evaluated on the aggressive B16 melanoma cancer model. The extract was subcutaneously administered to mice at doses of 2.5mg/kg, 1.25 mg/kg and 0.63mg/kg. The extract was administered daily, five days a week, for five consecutive weeks.

The data detailed in Table 7 show that the B16 tumor-bearing mice died before giving them the 25 injections of the extract, except for the 0.63 mg/kg dose. The number of injections when reaching the T/C index was 20 for the dose of 2.5mg/kg, 20 for the dose of 1.25mg/kg and 25 for the dose of 0.63mg/kg. Thus, especially the treatment at doses of 0.63 mg/kg induced a significant increase in the survival periods of B16 tumor-bearing mice.

TABLE 7 Number of dead mice upon administration of the extract according to the invention under the given conditions at several days after grafting

	doses	Number of injections at T/C	Days after grafting															
			21	22	23	25	28	29	30	31	35	39	42	46	51	53	56	63
EXTRACT	(5x5) 2.5mg/kg	20					1		2	1	1		1	1		1		1
	(5x5) 1.25mg/kg	20				1	1			1	2		1	1			1	1
	(5x5) 0.63mg/kg	25			1		1	1			1		2	1	1			1
CONTROL	5x5	17	1	1		1		1	1		1	3						

Figure 23A shows that the administered doses had negligible toxic effects since the B16-melanoma-bearing mice lost no body weight during the treatment. Figure 23B shows that the extract administrations dispensed at 2.5 or 1.25 mg/kg significantly decreased the growth of the B16 melanoma. In addition, the T/C index values for the 20 x 2.5 mg/kg administration schedule gave a T/C index of 117 %, the T/C index values for the 20 x 1.25 mg/kg schedule gave a T/C index of 117 % and the T/C index values for the 25 X 0.63mg/kg schedule gave a T/C index of 140%. Thus, the extract decreased the growth of the B16 melanoma and significantly prolonged the survival of the B16 melanoma-bearing mice, especially when the extract was administered 25 times at 0.63 mg/kg when the level of significance is reached.

In a second set of experiments, the extract was administered orally (*per os*). The data detailed in Table 8 below show that the B16 tumor-bearing mice died before giving them the 25 injections of the extract.

TABLE 8 Number of dead mice upon administration of the extract according to the invention under the given conditions at several days after grafting

	doses	Number of injections at T/C	Days after grafting										
			21	22	23	24	25	28	29	30	32	35	37
EXTRACT	(5x5) 2.5mg/kg	19			2			1	1		2	1	2
	(5x5) 1.25mg/kg	15	1	1		1		3	1		2		
	(5x5) 0.63mg/kg	16	1		1	1			2	1	1		2
CONTROL	5x5	15	1		1	1		1	1	1		2	

Figure 24A shows that the administered doses had negligible toxic effects since the B16-melanoma-bearing mice lost no body weight during the treatment. Figure 24B shows that the extract administrations *per os* at 2.5 or 1.25 mg/kg significantly decreased the growth of the B16 melanoma up to 25 days post-graft. In addition, the T/C index values for the 19 x 2.5 mg/kg administration schedule gave a T/C index of 114%, the T/C index values for the 15 x 1.25 mg/kg schedule gave a T/C index of 100% and the T/C index values for the16 x 0.63 mg/kg schedule gave a T/C index of 104%. Thus, the extract induced a decrease in the growth of the B16 melanoma tumor and slightly prolonged the survival of the B16 melanoma-bearing mice at 2.5mg/kg.

In conclusion the two sets of experiments provide evidence that the extract according to the invention exerts significant anti-tumor effects on the B16 melanoma model independent of the mode of administration.

In vivo effects of the extract according to the invention on a breast cancer model

The extract according to the invention was evaluated on the MXT-HI breast cancer model, i.e. a hormone insensitive variant of breast cancer. The MXT-HI model corresponds to an undifferentiated carcinoma with dramatic metastatic processes towards the liver. It therefore corresponds to the late clinical stages of human breast cancer. The MXT-HI represents a very aggressive biological tumor model.

The MXT-HI breast cancer is induced in mice by subcutaneously injecting MXT tumor fragments into the flanks of B6D2F1 mice. Without treatment, the inoculated mice die between the fourth and seventh week after the inoculation. The extract according to the invention was assayed at 10 mg/kg, 5 mg/kg and 2,5 mg/kg with nine administrations, i.e. three times a week for three consecutive weeks. The data detailed in Table 9 below show that the MXT-HI tumor-bearing mice survived the administration of the nine injections of the extract, and died later on during the experiment.

TABLE 9 Number of dead mice upon administration of the extract according to the invention under the given conditions at several days after grafting

	doses	Number of injections at T/C	Days after grafting									
			28	30	31	32	35	37	38	39	42	43
EXTRACT	(3x3) 10mg/kg	9	1		2	2	1		1		2	
	(3x3) 5 mg/kg	9		2	2	3	2					
	(3x3) 2.5 mg/kg	9		1			2	2	1	1		1
CONTROL	3x3	9	4		2		1	2				

Figure 25A indicates that the various extract treatment schedules used in the present experiments induced essentially no major toxic-side effects since the MXT-HI-bearing tumor mice did not lose significant weight. Figure 25B shows that nine administrations of 2.5 mg/kg significantly decreased MXT-HI tumor growth. The T/C index values for the 9 x 10 mg/kg administration schedule gave a T/C index of 103%, the T/C index values for the 9 x 5 mg/kg schedule gave a T/C index of 103% and the T/C index values for the 9 x 2.5 mg/kg schedule gave a T/C index of 119%. Thus at the dose of 2.5 mg/kg, the extract prolonged the survival of the MXT-HI-bearing mice.

In figure 26 the number of the MXT-HI breast cancer bearing mice, which died during the experiment in the control (untreated) and test groups (treated with extract), is shown. A Kaplan-Meier statistical analysis was used and represents the death rate of the nine mice in each group. The statistic value underlines the general fit of the test group in comparison with the general fit of the control group with regard to survival.

In conclusion the experiments described above provide evidence that the extract according to the invention exerts significant anti-tumor effects on the MXT-HI breast cancer tumor.

Conclusions on example 6

The extract according to the invention has a significant anti-tumor effect on the tested cancer models. These models represent a broad panel of histological tumor types, including carcinomas, lymphomas and melanomas. These models are clinically relevant because they mimic specific clinical stages of human cancers. Apart from this, they are also biologically aggressive and invasive because two of them metastasize dramatically to the liver.

While having a very significant anti-tumor effect, the extract according to the invention exhibits negligible toxic effects since the tumor-bearing mice did not lose body weight during the treatments. The semi-purified extract can therefore include an "antidote" in addition to the anti-tumor compound responsible for all the anti-tumor activities reported here.

Surprisingly, the extract induces significantly higher anti-tumor activities when assayed chronically at low doses i.e. around 1.25 to 0.63mg/kg, than at high doses, i.e. around 10mg/kg to 5 mg/kg.

Example 7 In vivo toxicological effects of the extract according to the invention

This example illustrates the toxicological effects of the extract according to the invention, in particular the *in vivo* hematotoxicity and general toxicity.

Hematotoxicity

Hematotoxicity is evaluated by establishing hematological profiles for each animal species. Particular attention was paid to the numbers of blood platelets, red cells and leukocytes. The effect of the extract according to the invention was evaluated at two doses, i.e. 5 mg/kg and 1.25 mg/kg, by the intra-peritoneal administration to mice. The administration schedule was five administrations a week for five consecutive weeks resulting in a total injection number of twenty-five. The animals were sacrificed three days after the last injection. There were ten mice per group.

As compared to control (Figure 27), no statistically significant changes were observed with respect to hematological parameters after treatment with the extract according to the invention. No significant changes were observed on the mean cell volume of red blood cells,

the mean corpuscular hemoglobin, the mean corpuscular hemoglobin concentration and the platelets after the treatments.

General toxicity

The general toxicity of the extract according to the invention was histologically tested on mice by means of conventional histopathological analyses of hematoxilin-eosin-stained histological slides obtained from different types of organs and tissues. The effect of the extract was evaluated at 5 mg/kg and at 1.25 mg/kg by intra-peritoneal administration. The administration schedule was five administrations a week for five consecutive weeks resulting in a total injection number of twenty-five. The animals were sacrificed three days after the last injection. The brain, the heart, the liver, the pancreas, the stomach, the intestines and the ovaries were collected. There were ten mice per group.

Examination of the collected organs showed that control (untreated) group did not show any particular modification in the organs. Also in treated mice the brain, the heart, the liver, the pancreas, the stomach, the intestines and the ovaries were not affected by the extract treatments at doses of 5mg/kg or 1.25 mg/kg, indicating that the extract according to the invention does not have a general toxic effect.

Example 8 Anti-poisonous effects of the extract according to the invention

This example illustrates the anti-poisonous effect of the extract according to the invention. Female mice of 4 to 5 weeks of age were injected with two times the maximal tolerable dose (MTDx2) of two well-known and clinically used anti-tumor drugs: adriamycine and vincristine. The mice were given a single intraperitoneal injection with 10mg/kg body weight adriamycine or a single intraperitoneal injection of 20 mg/kg body weight vincristine at Day 0. Lot 1 in figure 28 shows the survival curve of the mice injected with the MTDx2 of either vincristine or either adriamycine.

The extract according to the invention was injected intraperitoneally at a dose of 10 mg/kg body weight prior to the injection of the anti-tumor drugs. The following different schedules were applied.

- The extract was injected on the same day (i.e. at Day 0), but 4 hours prior to the injection of the cytotoxic drug adriamycine or vincristine. Results of this treatment are represented by lot 2, in figure 28.

- The extract was injected once a day during eight days prior to the day of injecting the cytotoxic drug adriamycine or vincristine. Results of this treatment are represented by lot 3, in figure 28.
- The extract was injected once a day during eight days prior to the day of injecting the cytotoxic drug adriamycine or vincristine and was further once a day during seven days after the injection of the anti-tumor drugs. Results of this treatment are represented by lot 4, in figure 28.

The parameter, which was applied for determining the protective, i.e. anti-poisonous effect of the extract according to the invention, on the toxic effects induced by the anti-tumor drugs consisted of the "prolongation of survival". Statistical analysis was performed by means of Kaplan-Meier statistics. The level of significance was $p<0.05$.

As can be seen in figure 28, all schedules wherein the extract is used in combination with an cytotoxic drug significantly prolong the survival of the mice as compared to injection of the cytotoxic drugs as single agents. It can thus be concluded that the extract according to the invention enables to significantly protect mice from the toxic effects of a mortal dose of frequently used anti-tumor drugs such as adriamycine or vincristine.

In conclusion, this experiment illustrates that the extract according to the invention has an anti-poisonous effect, which enables to reduce the toxic effects of well-known anti-tumor drugs.

Claims

1. An extract of the plant *Calotropis procera,* characterized in that said extract has a pharmacological activity, in particular an anti-tumor activity.

2. An extract according to claim 1, whereby said extract exerts an anti-tumor activity at low doses in the range of 0.5 to 2 mg/kg.

3. An extract of the plant *Calotropis procera* according to claim 1 or 2, characterized in that said extract exhibits a low toxicity level.

4. An extract of the plant *Calotropis procera* according to claim 1, characterized in that said extract further exhibits an anti-poisonous activity.

5. An extract of the plant *Calotropis procera* according to claim 1 to 4, comprising at least two active compounds selected from the group comprising asclepin, calactin, vorusharin, calotropin, calotropagenin, uzarigenin, calotoxin, usharin and usharidin.

6. An extract according to any of claims 1 to 5, whereby said extract further contains at least one of the compounds, which are represented in Table 1.

7. Active compound isolated from the extract according to any of claims 1 to 6.

8. Extraction process for obtaining an extract according to any of claims 1 to 6, comprising the steps of:

a) extracting the starting material of said *Calotropis procera* plant, said starting material being selected among fruits, aerial parts, subterranean parts, and their mixtures, in an aliphatic alcohol, by dissolving the starting material in said alcohol thereby obtaining a suspension of said material in said alcohol, stirring said suspension; and filtering said suspension by fritted glass thereby obtaining a first filtrate and a first solid part;

b) extracting said first solid part in an aliphatic alcohol thereby obtaining a second filtrate and a second solid part;

c) combining said first and said second filtrate thereby obtaining a combined filtrate; and

d) evaporating said combined filtrate under vacuum thereby obtaining an oily residue.

9. Use of an extract according to any of claims 1 to 6, or an active compound thereof according to claim 7 as a medicament.

10. Use of an extract according to any of claims 1 to 6, or an active compound thereof according to claim 7 for the preparation of a medicament in the treatment of cancer.

11. Use of an extract or an active compound thereof according to claim 10 wherein said cancer is selected from the group comprising breast cancer, lymphoma, sarcoma, pancreatic cancer, melanoma, colorectal cancer, glioma, lung cancer, bladder cancer, head and neck cancer, prostate cancer, liver cancer, hematological cancers.

12. A pharmaceutical composition for the treatment of cancer comprising a therapeutic effective amount of the extract of *Calotropis procera* according to any of claims 1 to 6, or an active compound thereof according to claim 7, and a pharmaceutical acceptable carrier.

13. A pharmaceutical composition comprising a first component, said first component being the extract of *Calotropis procera* according to any of claims 1 to 6, or an active compound thereof according to claim 7, and a second component, said second component comprising a therapeutic compound, which exerts a pharmacological activity having toxic side effects.

14. A method for treating cancer comprising administering to an individual in need of such treatment a pharmaceutical composition according to claims 12 or 13.

15. A method according to claim 14, wherein said cancer is selected from the group comprising breast cancer, lymphoma, sarcoma, pancreatic cancer, melanoma, colorectal cancer, glioma, lung cancer, bladder cancer, head and neck cancer, prostate cancer, liver cancer, hematological cancers.

Extract with anti-tumor and anti-poisonous activity

Abstract

The present invention relates an extract of the plant *Calotropis procera,* having a pharmacological activity, in particular an anti-tumor activity and/or an anti-poisonous activity and active compounds isolated thereof. Furthermore, the invention relates to a method for the extraction of said extract. Also, the invention concerns a pharmaceutical composition for the treatment of cancer comprising an effective amount of said extract or an active compound thereof and a pharmaceutical acceptable carrier. The present invention further relates to the use of said extract or an active compound thereof as a medicament and to the use of said extract or an active compound thereof for the preparation of a medicament for the treatment of cancer.

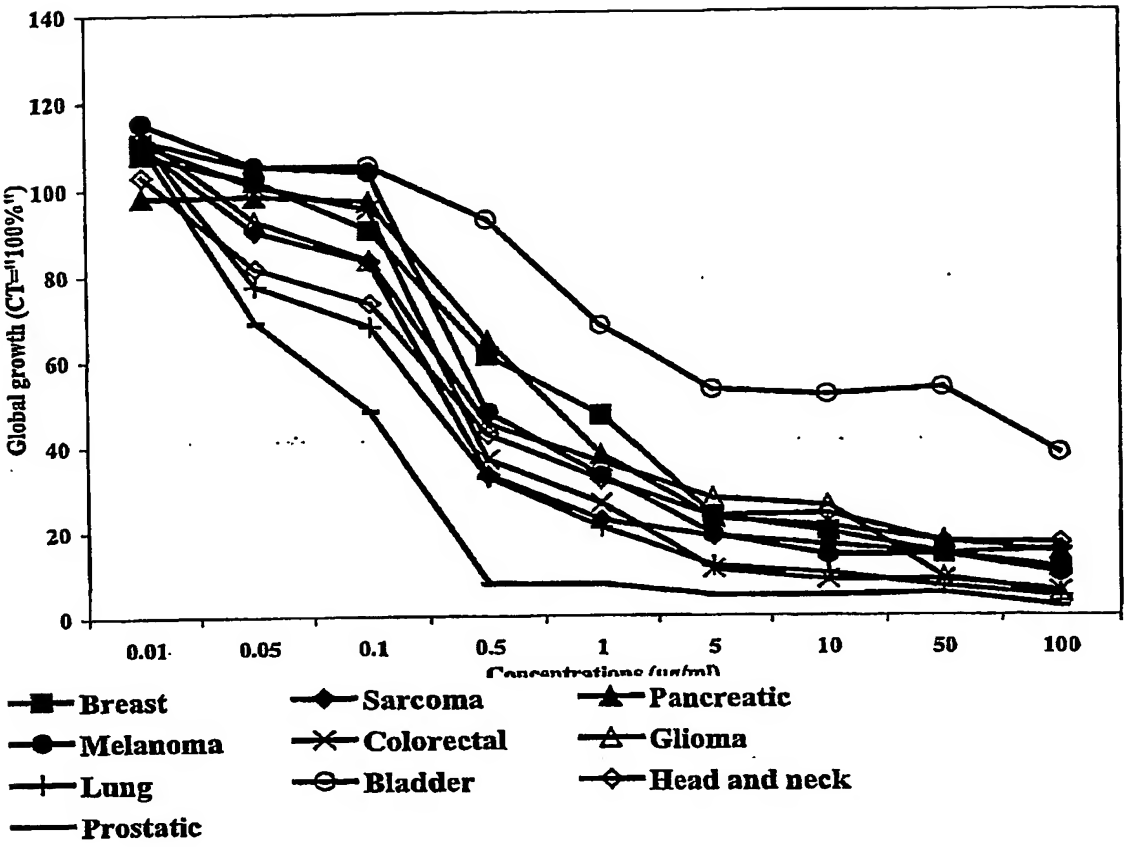

140
120
100
80
60
40
20
0
Global growth (CT="100%")
0.01
0.05
0.1
0.5
1
5
10
50
100
Breast
Sarcoma
Pancreatic
Melanoma
Colorectal
Glioma
Lung
Bladder
Head and neck
Prostatic

FIG. 1

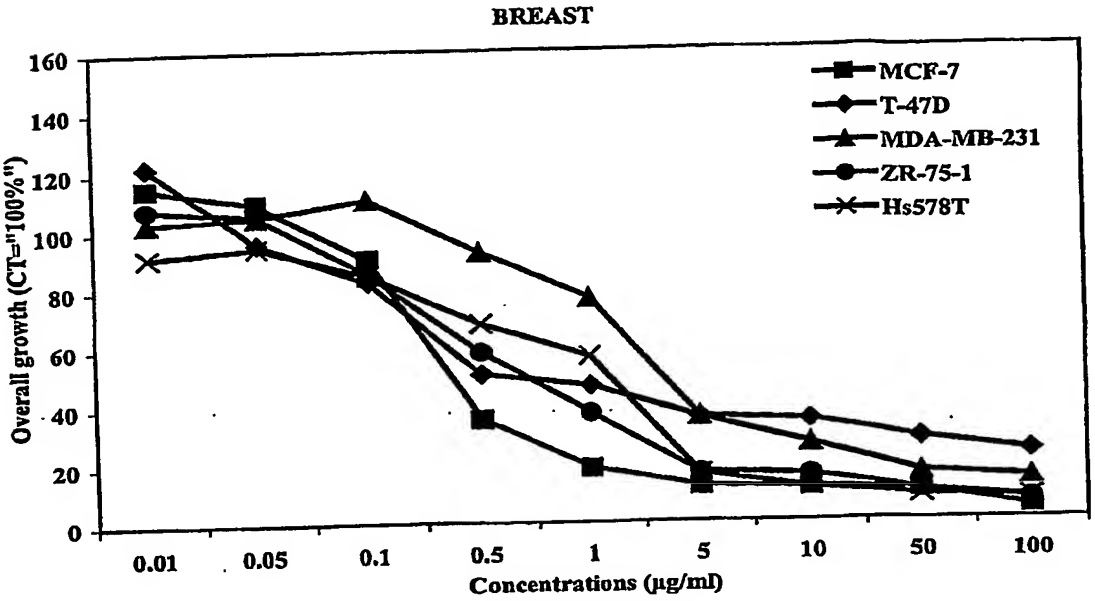

BREAST
Overall growth (CT="100%")
Concentrations (µg/ml)
MCF-7
T-47D
MDA-MB-231
ZR-75-1
Hs578T
0
20
40
60
80
100
120
140
160
0.01
0.05
0.1
0.5
1
5
10
50
100

FIG. 2

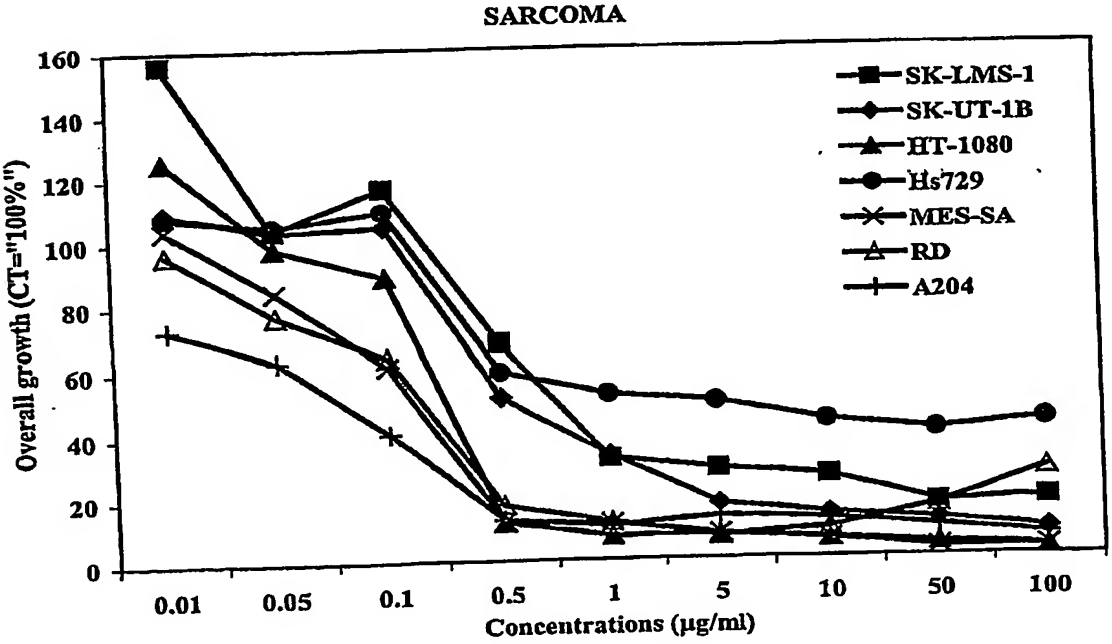

SARCOMA
Overall growth (CT="100%")
Concentrations (µg/ml)
SK-LMS-1
SK-UT-1B
HT-1080
Hs729
MES-SA
RD
A204
0
20
40
60
80
100
120
140
160
0.01
0.05
0.1
0.5
1
5
10
50
100

FIG. 3

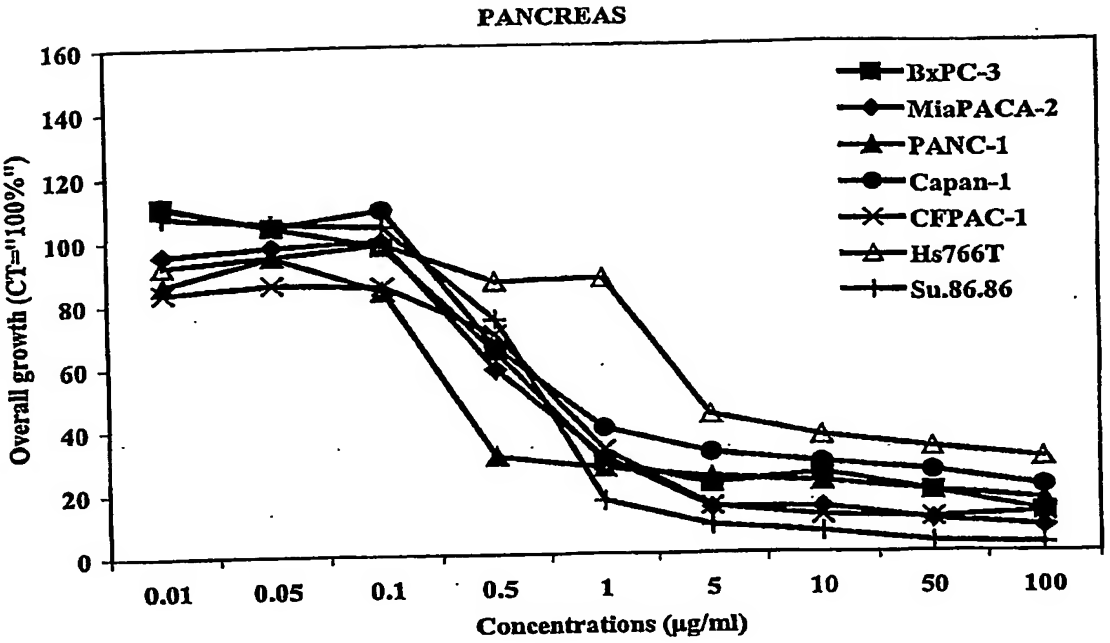

PANCREAS
Overall growth (CT="100%")
Concentrations (µg/ml)
BxPC-3
MiaPACA-2
PANC-1
Capan-1
CFPAC-1
Hs766T
Su.86.86
0
20
40
60
80
100
120
140
160
0.01
0.05
0.1
0.5
1
5
10
50
100

FIG. 4

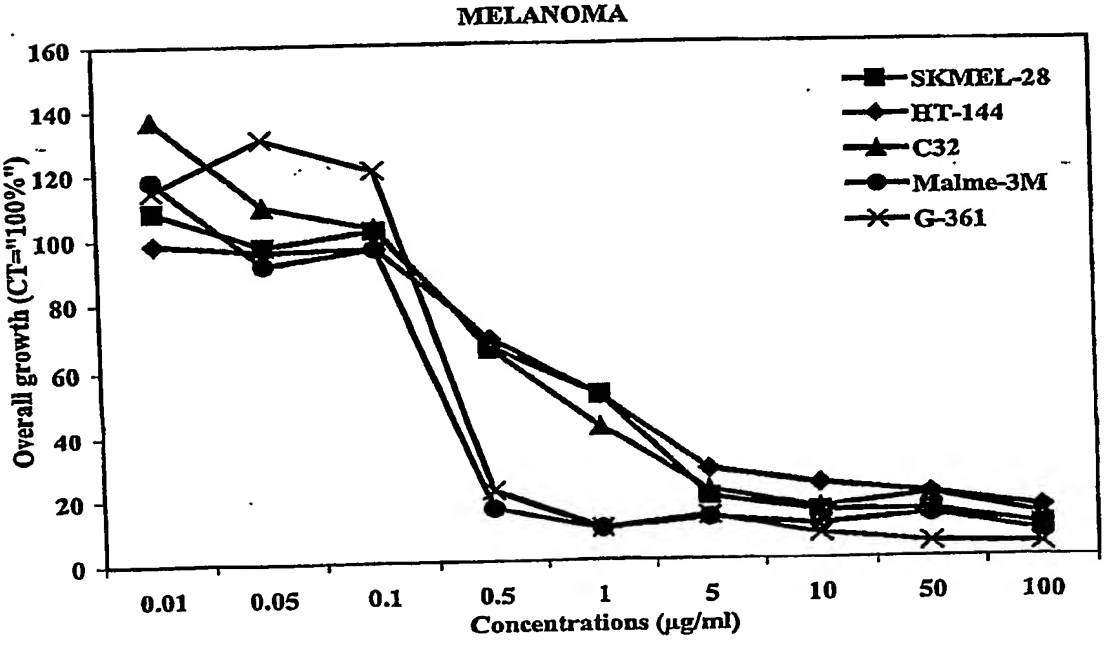

MELANOMA
Overall growth (CT="100%")
Concentrations (µg/ml)
SKMEL-28
HT-144
C32
Malme-3M
G-361
0
20
40
60
80
100
120
140
160
0.01
0.05
0.1
0.5
1
5
10
50
100

FIG. 5

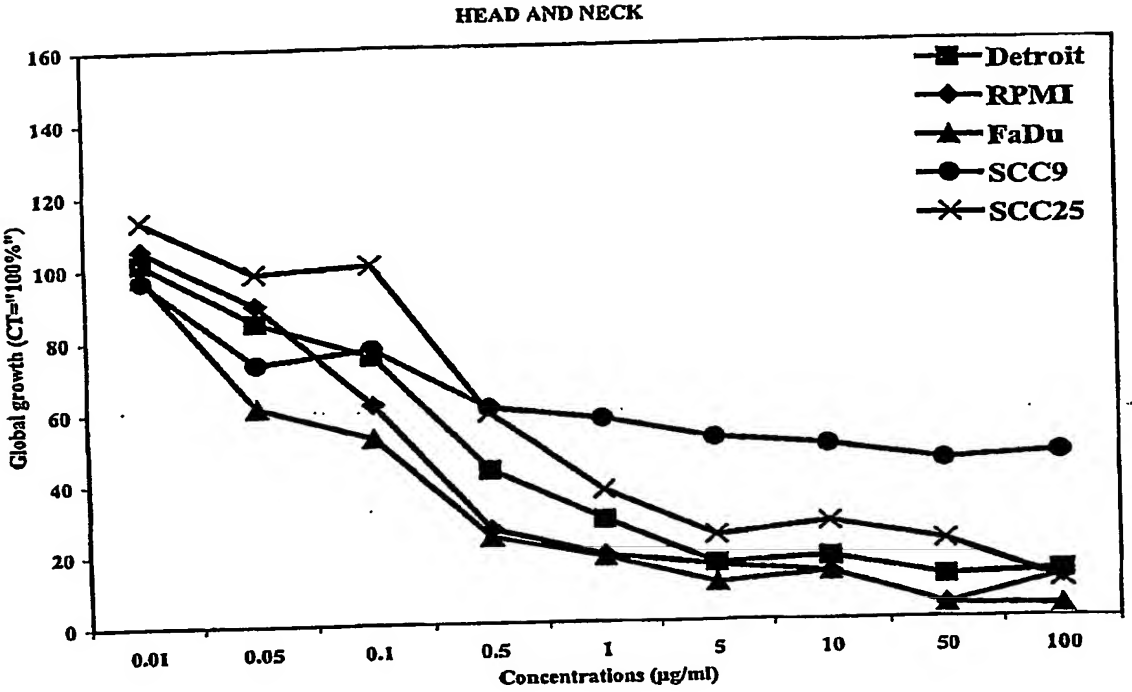

HEAD AND NECK
Detroit
RPMI
FaDu
SCC9
SCC25
Global growth (CT="100%")
160
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
5
10
50
100
Concentrations (µg/ml)

Fig. 6

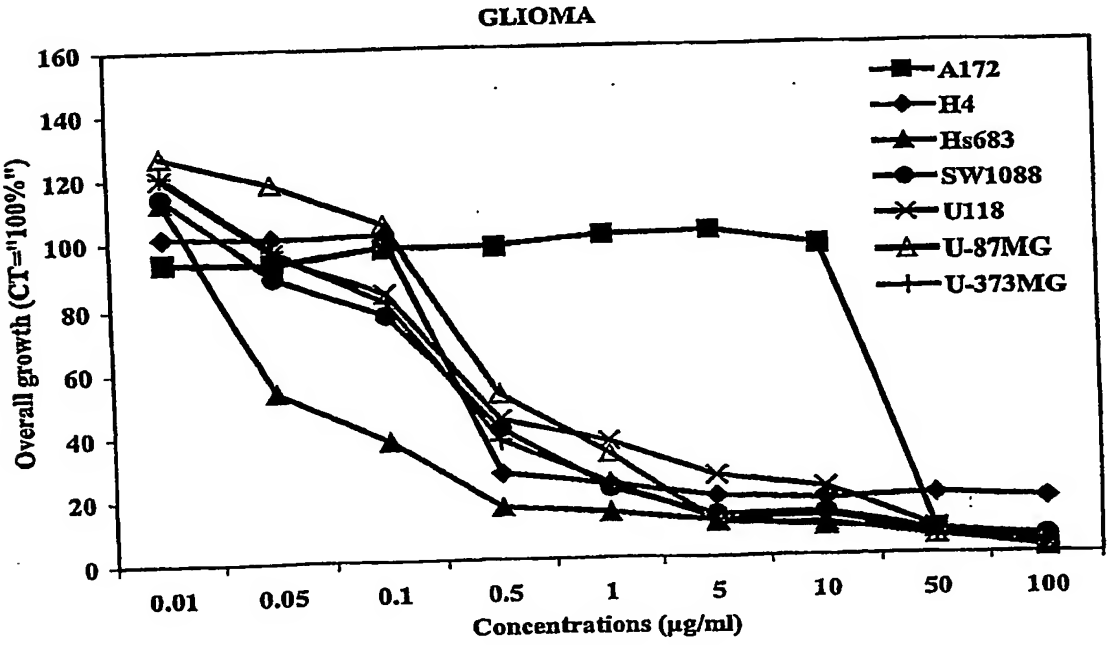

GLIOMA
A172
H4
Hs683
SW1088
U118
U-87MG
U-373MG
Overall growth (CT="100%")
160
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
5
10
50
100
Concentrations (µg/ml)

FIG. 7

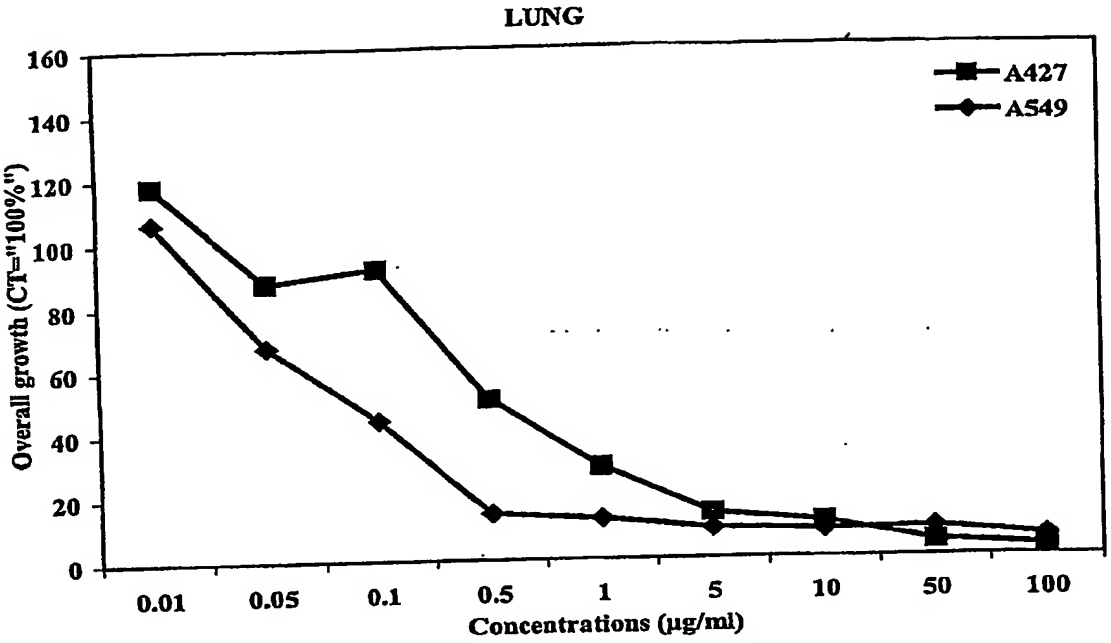

LUNG
A427
A549
Overall growth (CT="100%")
160
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
5
10
50
100
Concentrations (µg/ml)

FIG. 8

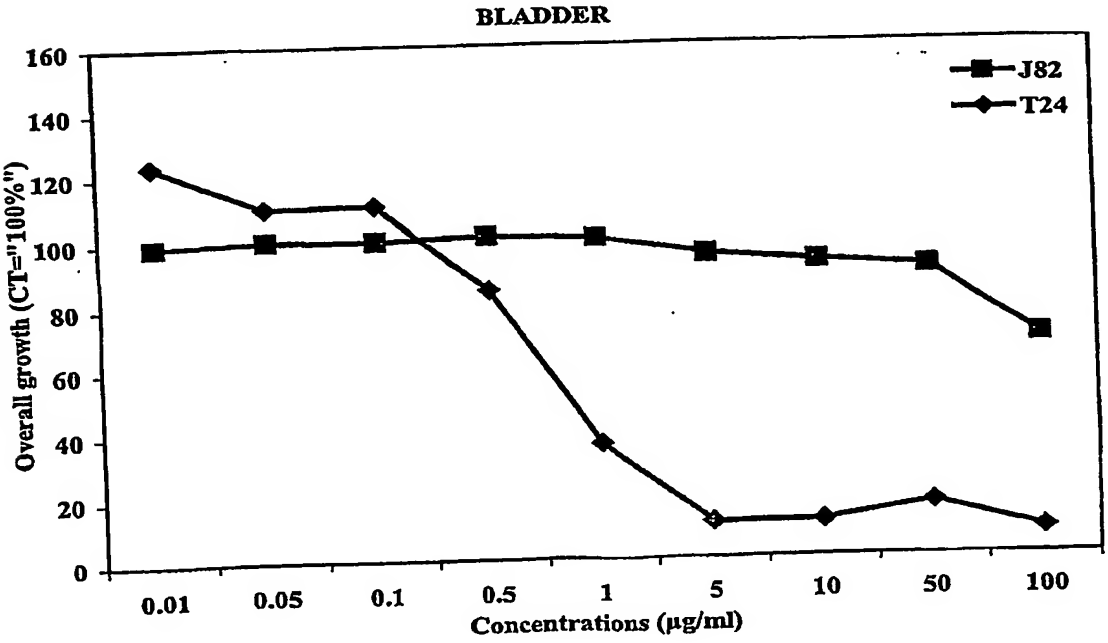

BLADDER
J82
T24
Overall growth (CT="100%")
160
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
5
10
50
100
Concentrations (µg/ml)

FIG. 9
6/23

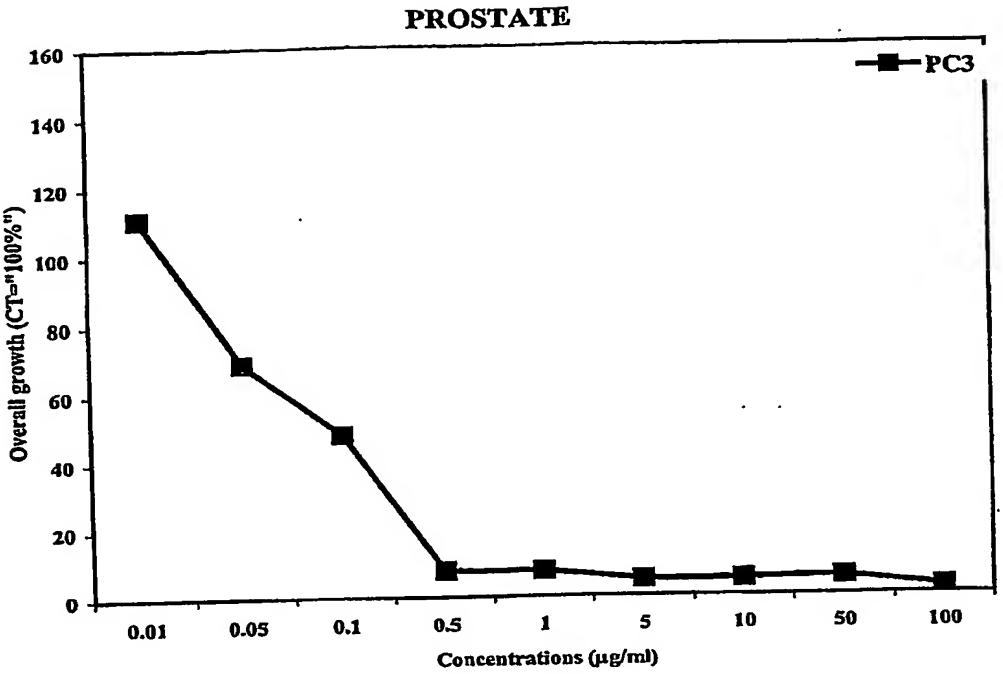


FIG. 10

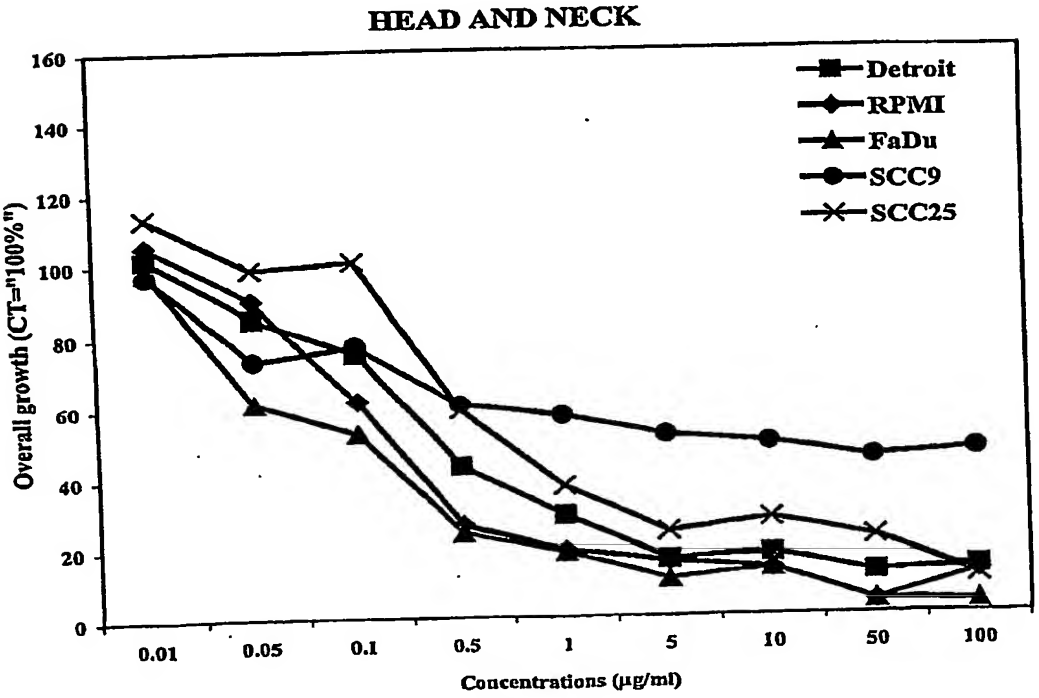
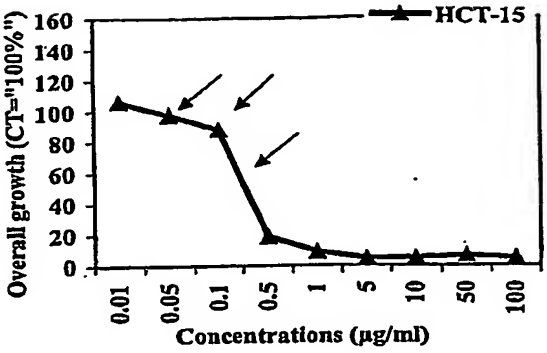


FIG. 11


HCT-15
Overall growth (CT="100%")
160
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
5
10
50
100
Concentrations (µg/ml)

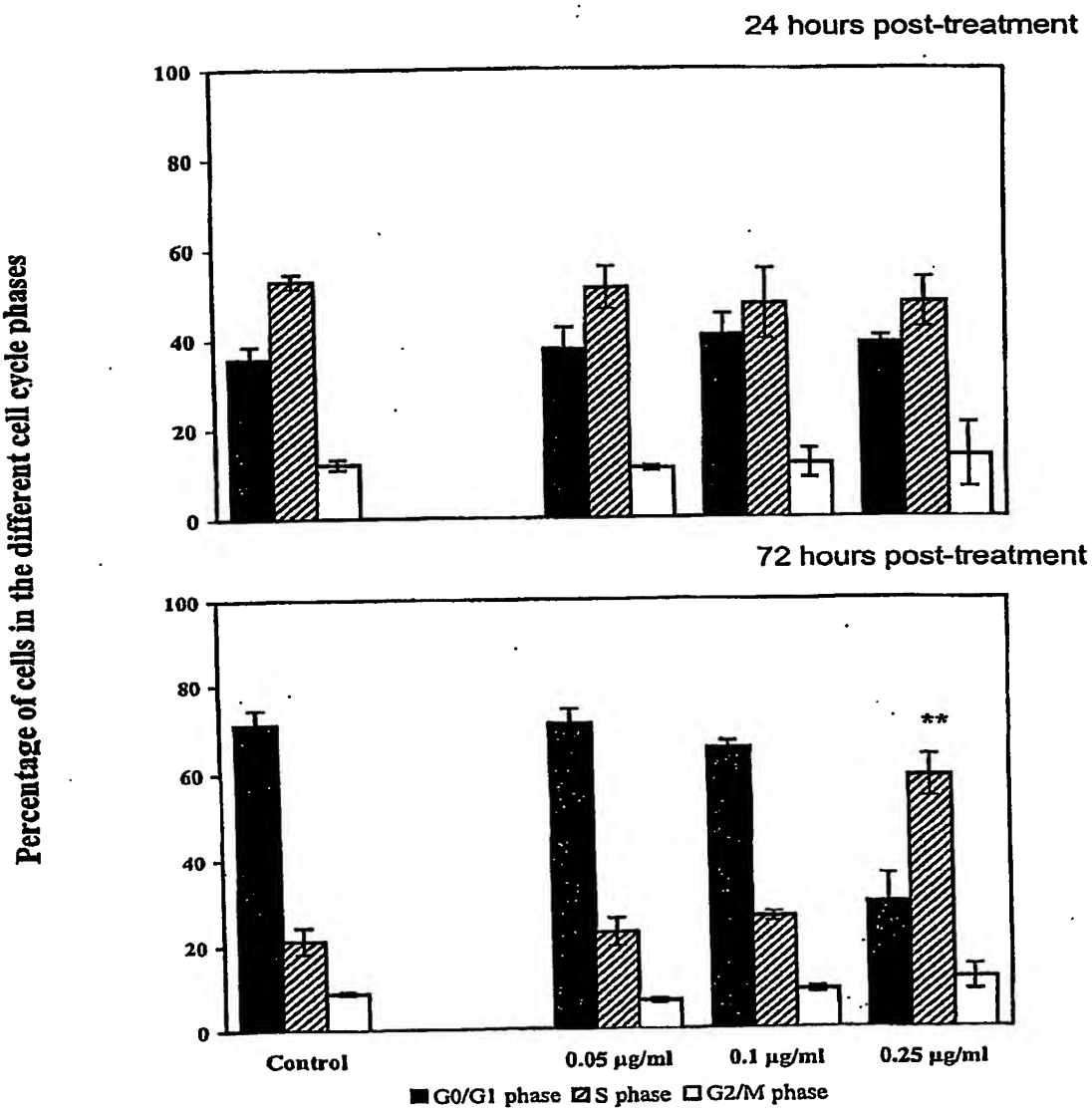
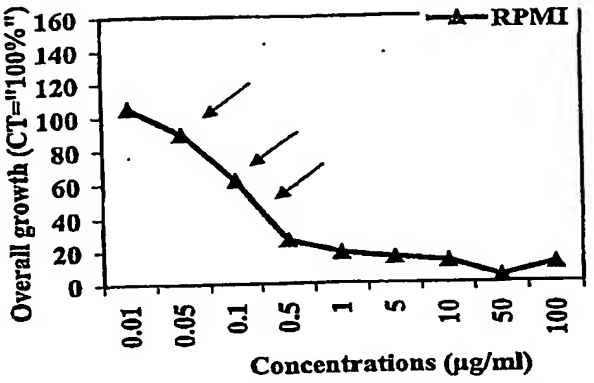

24 hours post-treatment
72 hours post-treatment
Percentage of cells in the different cell cycle phases
100
80
60
40
20
0
**
Control
0.05 µg/ml
0.1 µg/ml
0.25 µg/ml
G0/G1 phase
S phase
G2/M phase

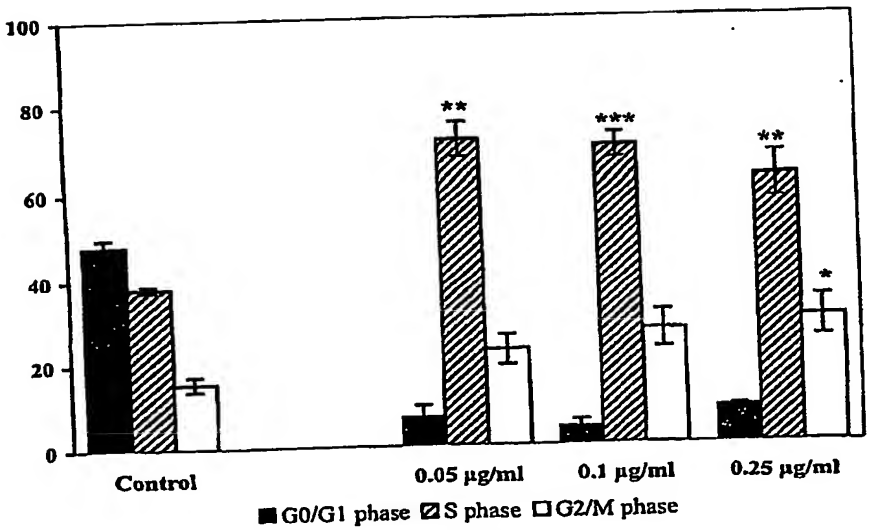
FIG. 12

8/23



24 hours post-treatment

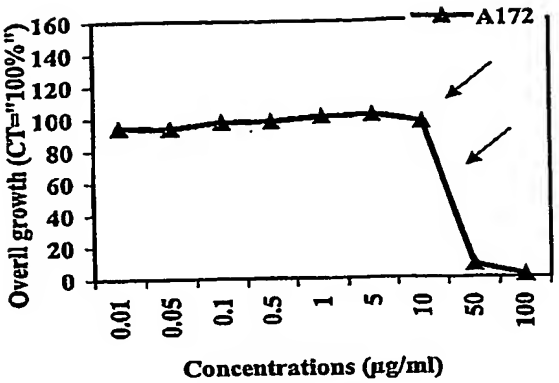
72 hours post-treatment



Percentage of cells in the different cell cycle phases

FIG. 13

9/23



24 hours Post-treatment

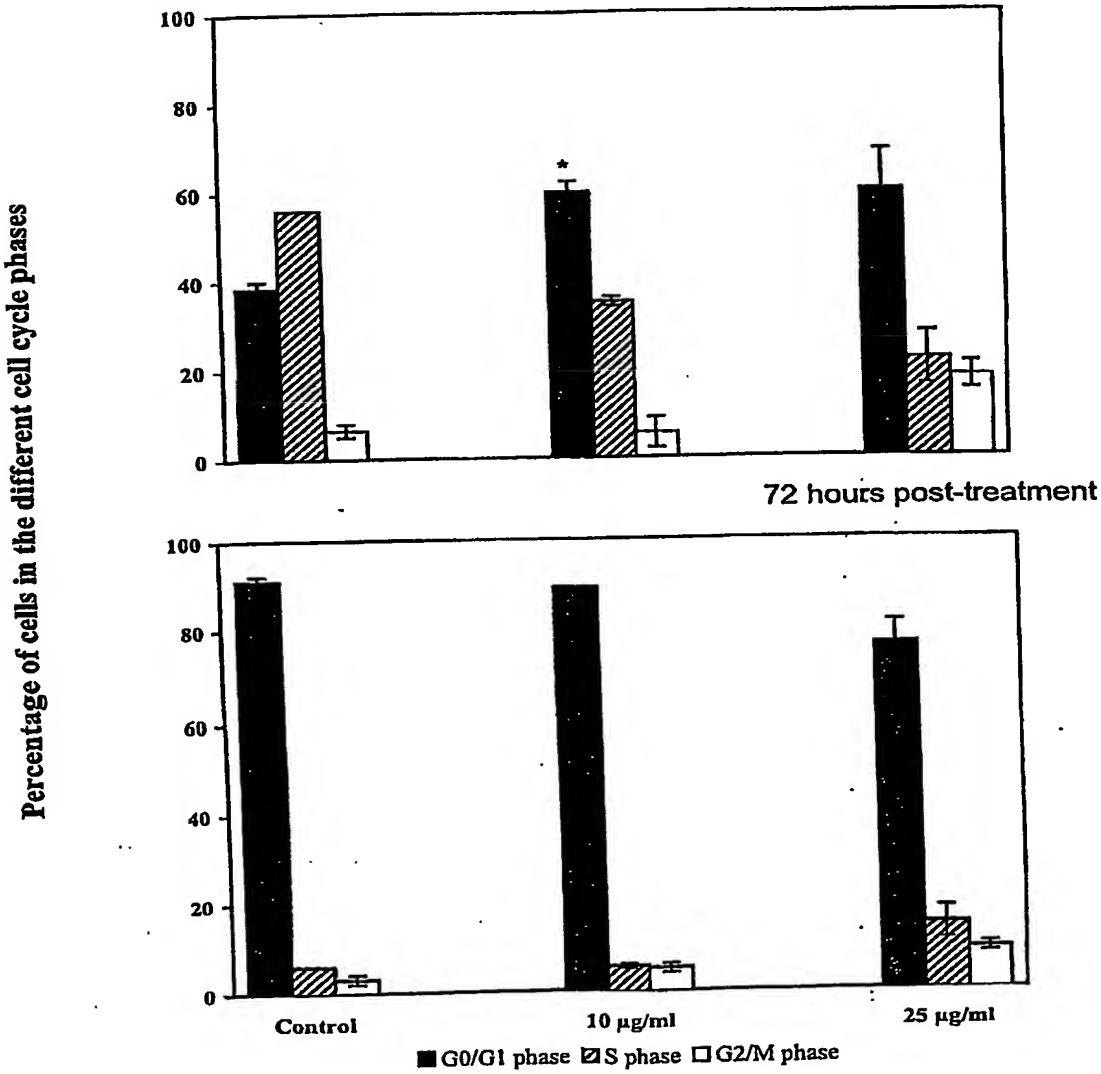


FIG. 14

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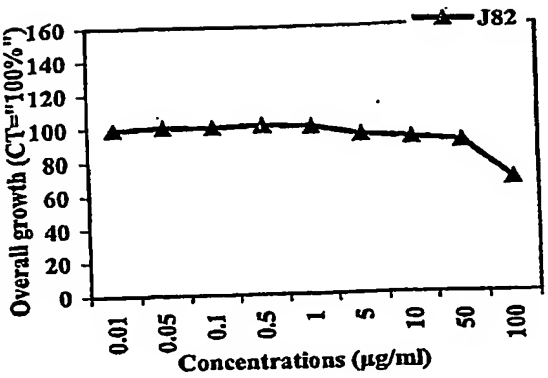
J82
Overall growth (CT="100%")
160
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
5
10
50
100
Concentrations (µg/ml)

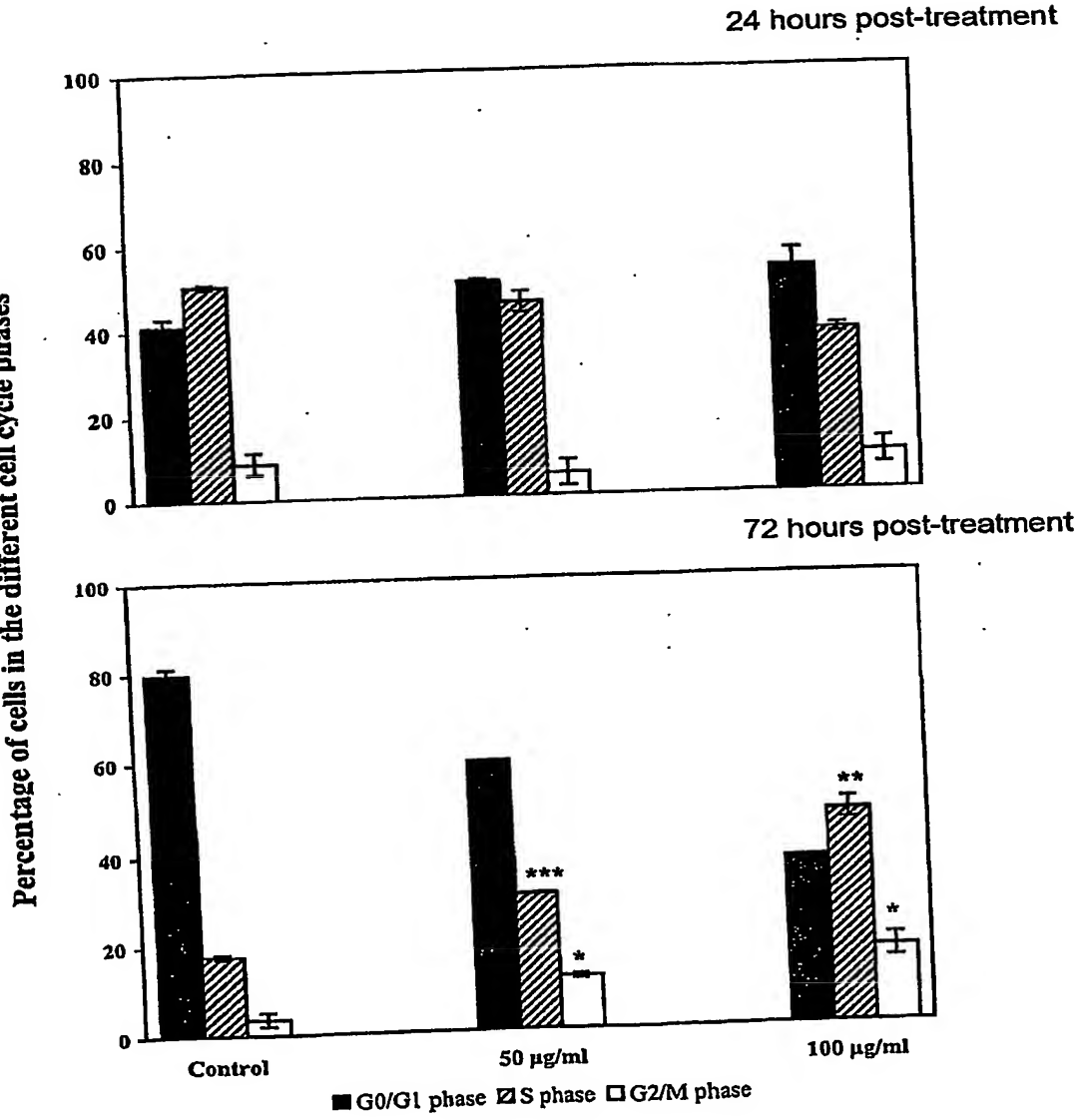
24 hours post-treatment
72 hours post-treatment
Percentage of cells in the different cell cycle phases
100
80
60
40
20
0
***
*
**
*
Control
50 µg/ml
100 µg/ml
G0/G1 phase
S phase
G2/M phase

FIG. 15

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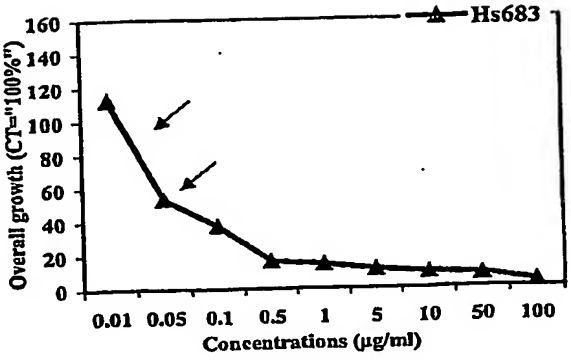


24 hours post-treatment

72 hours post-treatment

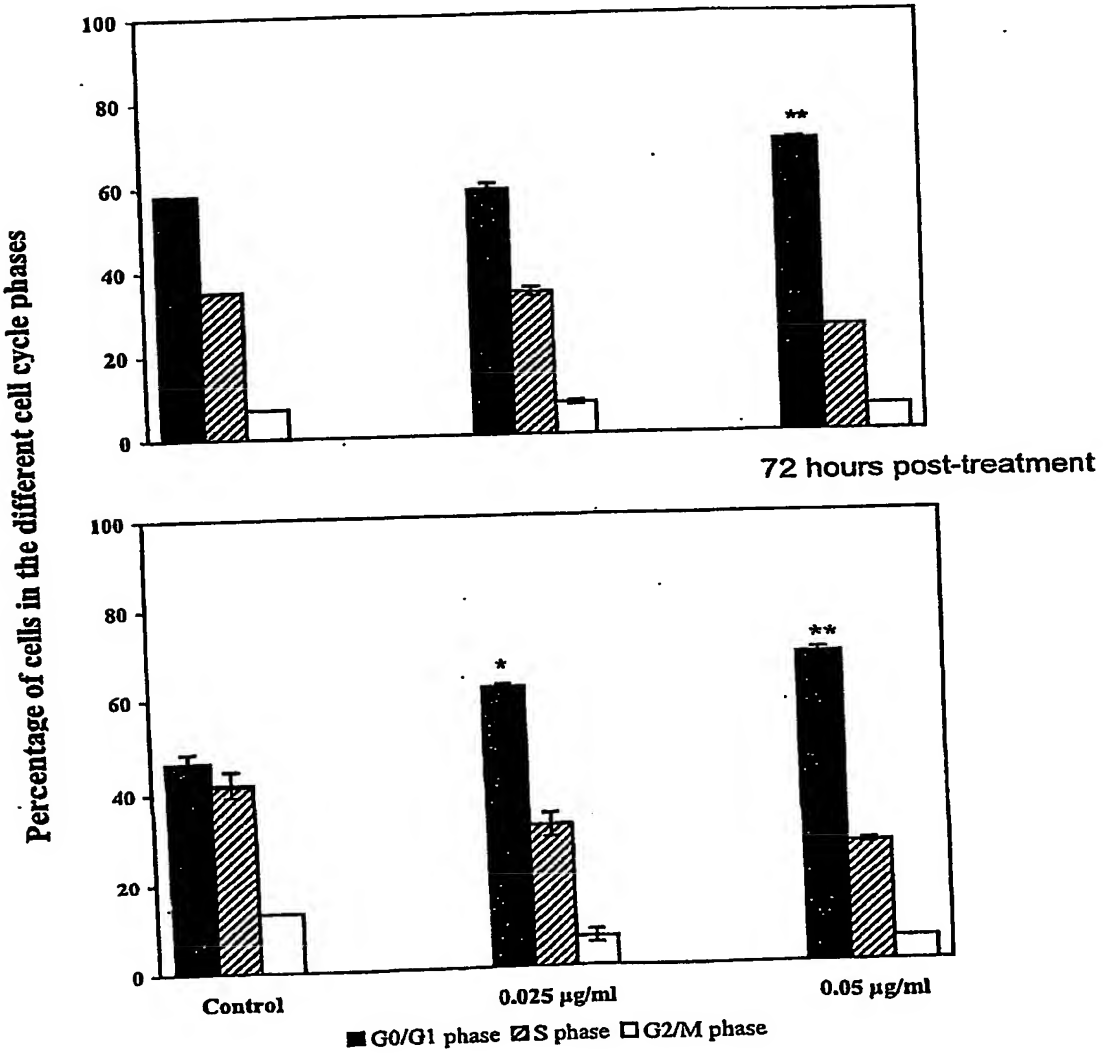
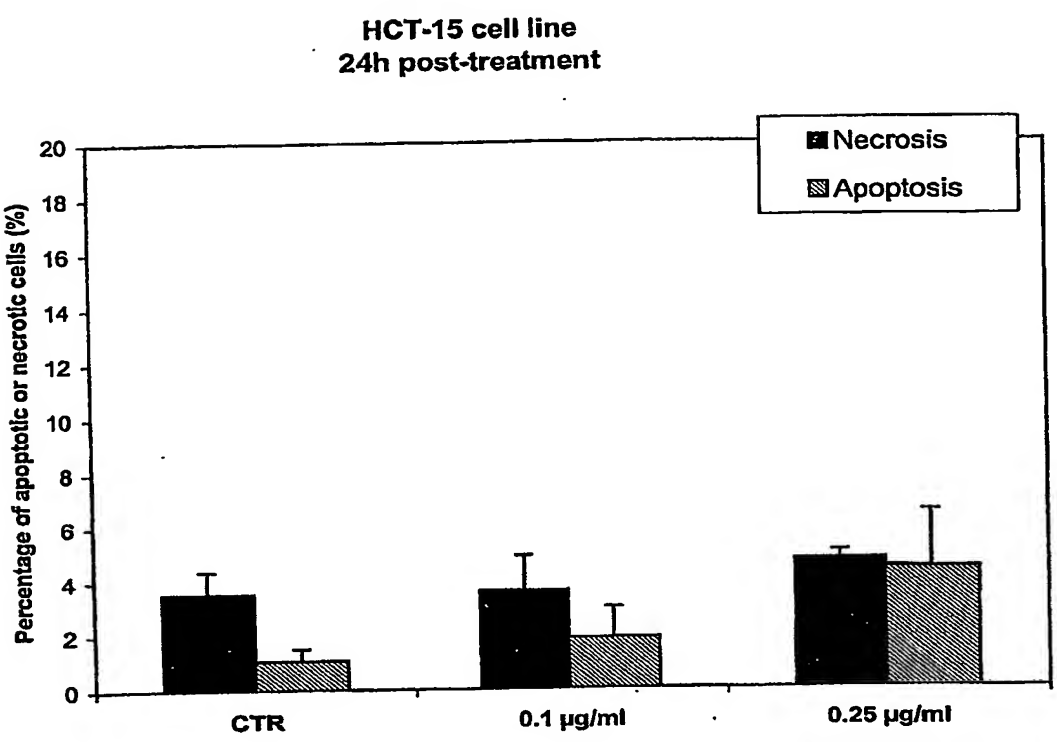


FIG. 16


HCT-15 cell line
24h post-treatment
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells (%)
0
2
4
6
8
10
12
14
16
18
20
CTR
0.1 µg/ml
0.25 µg/ml

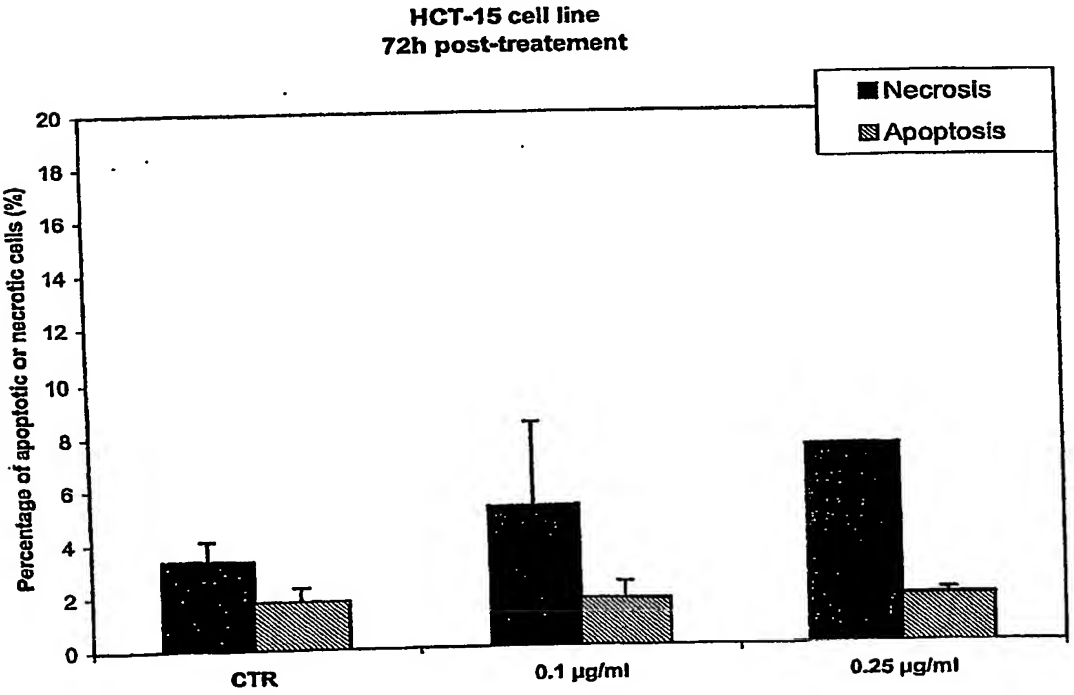

HCT-15 cell line
72h post-treatement
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells (%)
0
2
4
6
8
10
12
14
16
18
20
CTR
0.1 µg/ml
0.25 µg/ml

FIG. 17

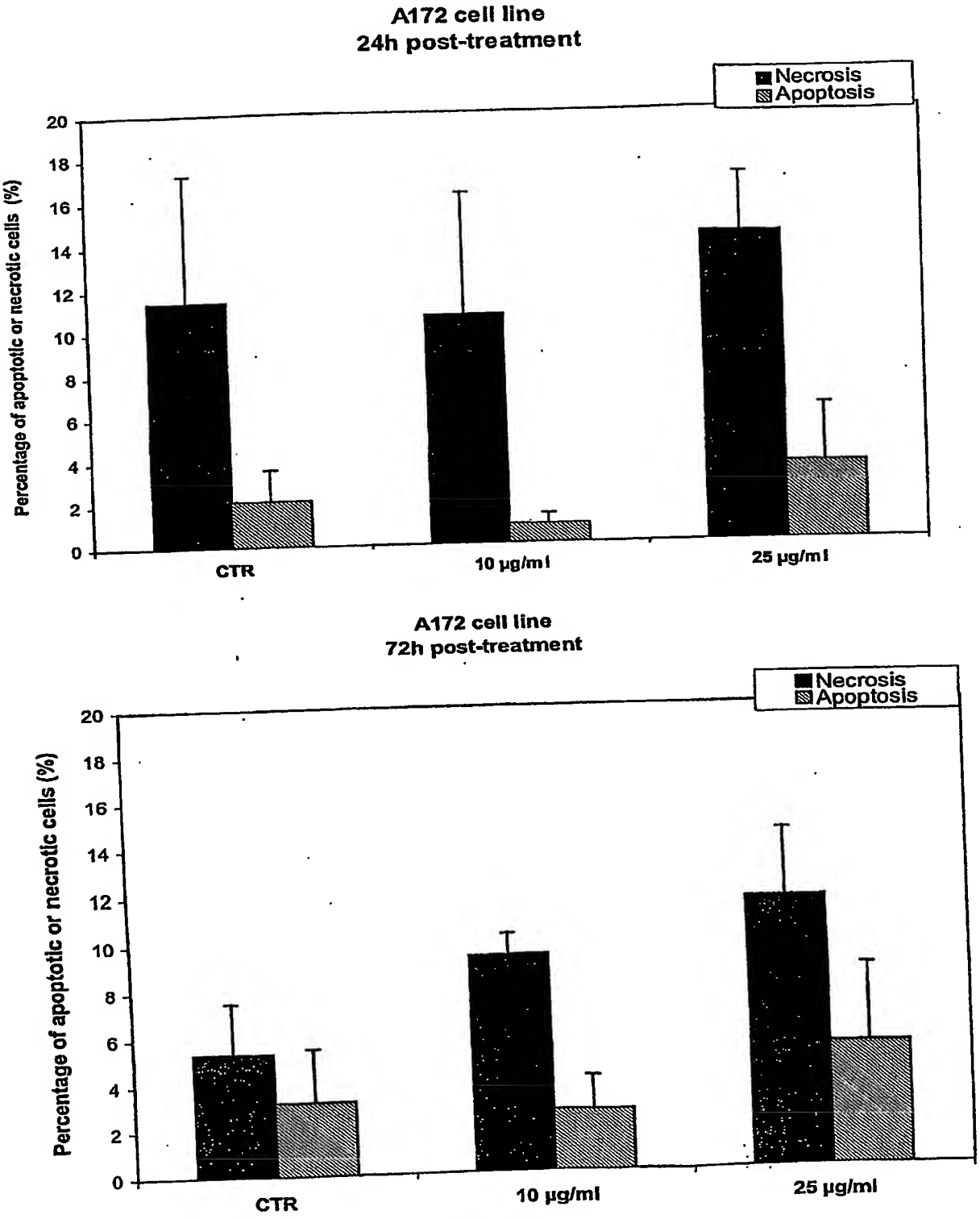
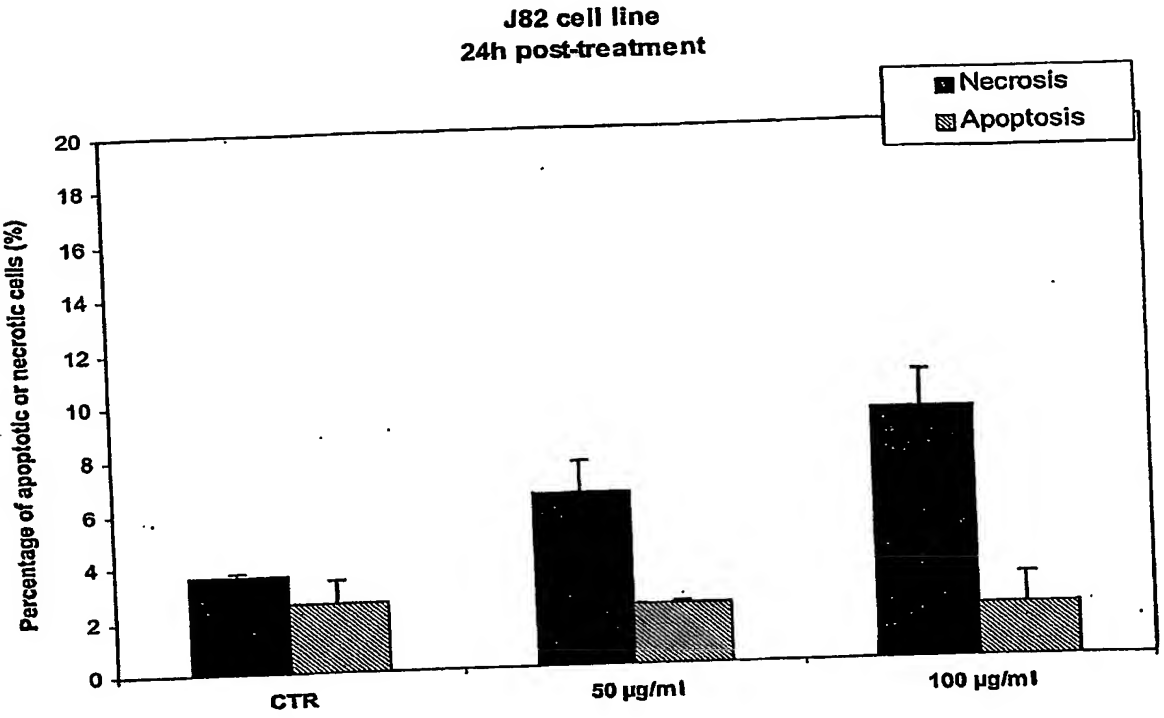

A172 cell line
24h post-treatment
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells (%)
0
2
4
6
8
10
12
14
16
18
20
CTR
10 µg/ml
25 µg/ml
A172 cell line
72h post-treatment
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells (%)
0
2
4
6
8
10
12
14
16
18
20
CTR
10 µg/ml
25 µg/ml

FIG. 18


J82 cell line
24h post-treatment
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells (%)
0
2
4
6
8
10
12
14
16
18
20
CTR
50 µg/ml
100 µg/ml

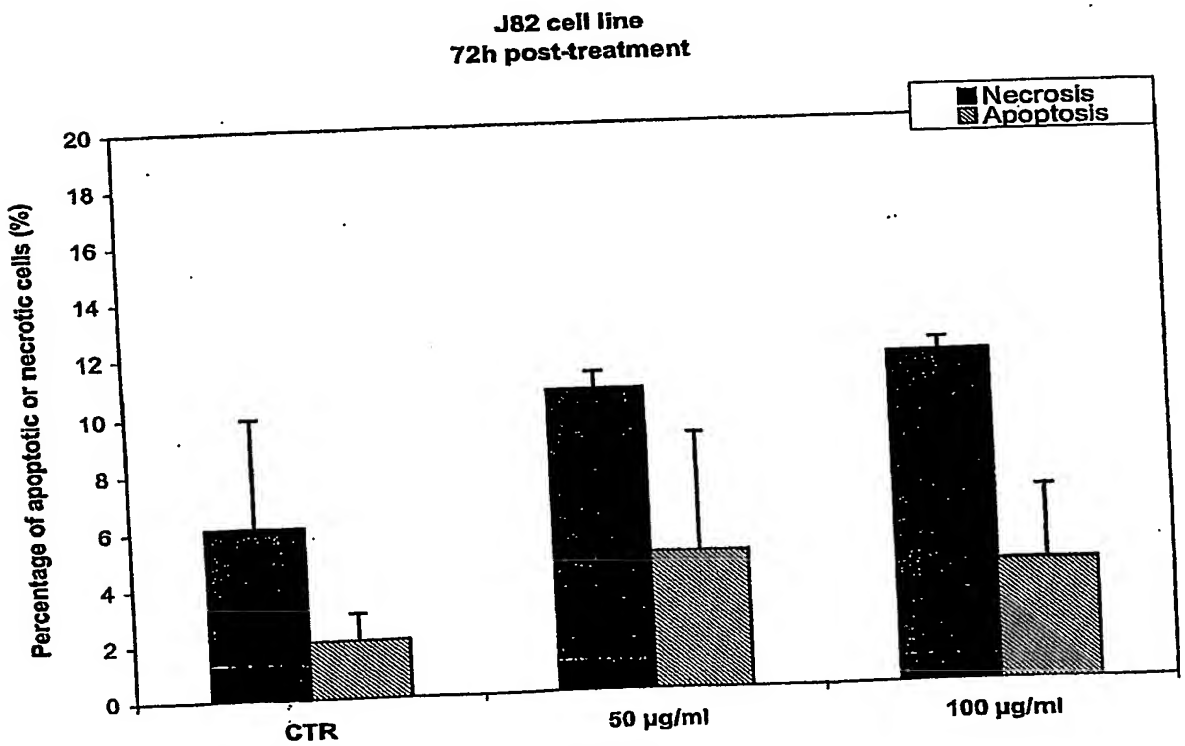
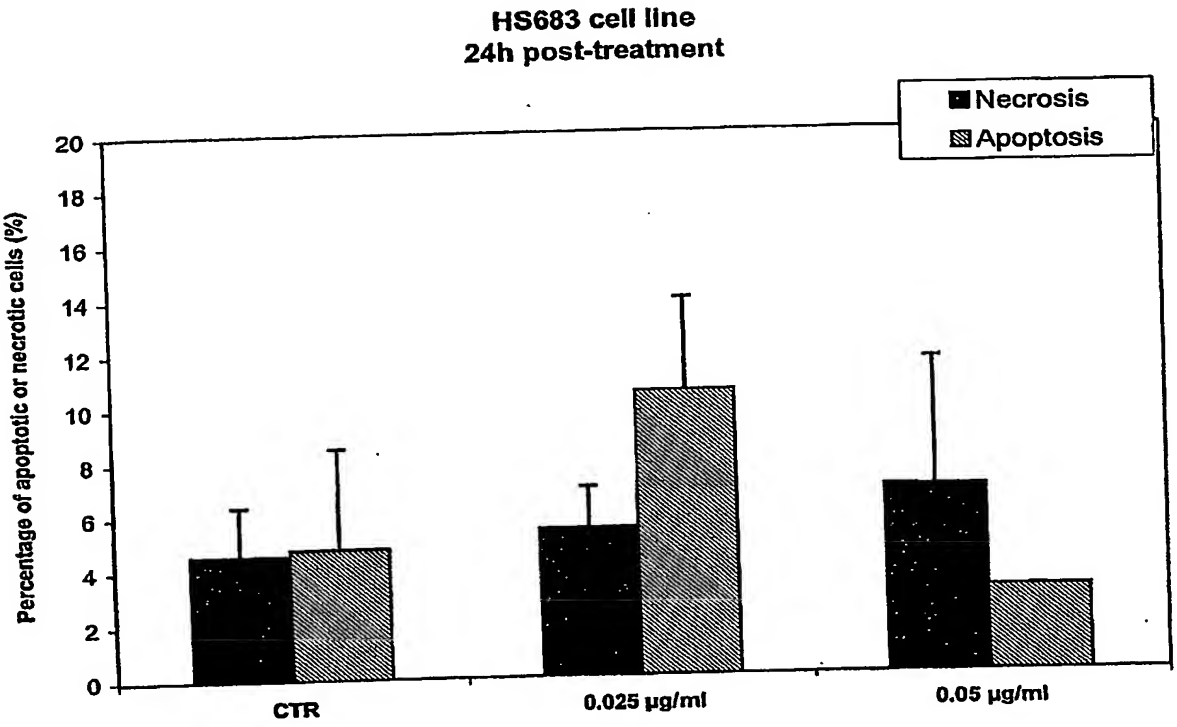

J82 cell line
72h post-treatment
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells (%)
0
2
4
6
8
10
12
14
16
18
20
CTR
50 µg/ml
100 µg/ml

FIG. 19


HS683 cell line
24h post-treatment
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells (%)
0
2
4
6
8
10
12
14
16
18
20
CTR
0.025 µg/ml
0.05 µg/ml

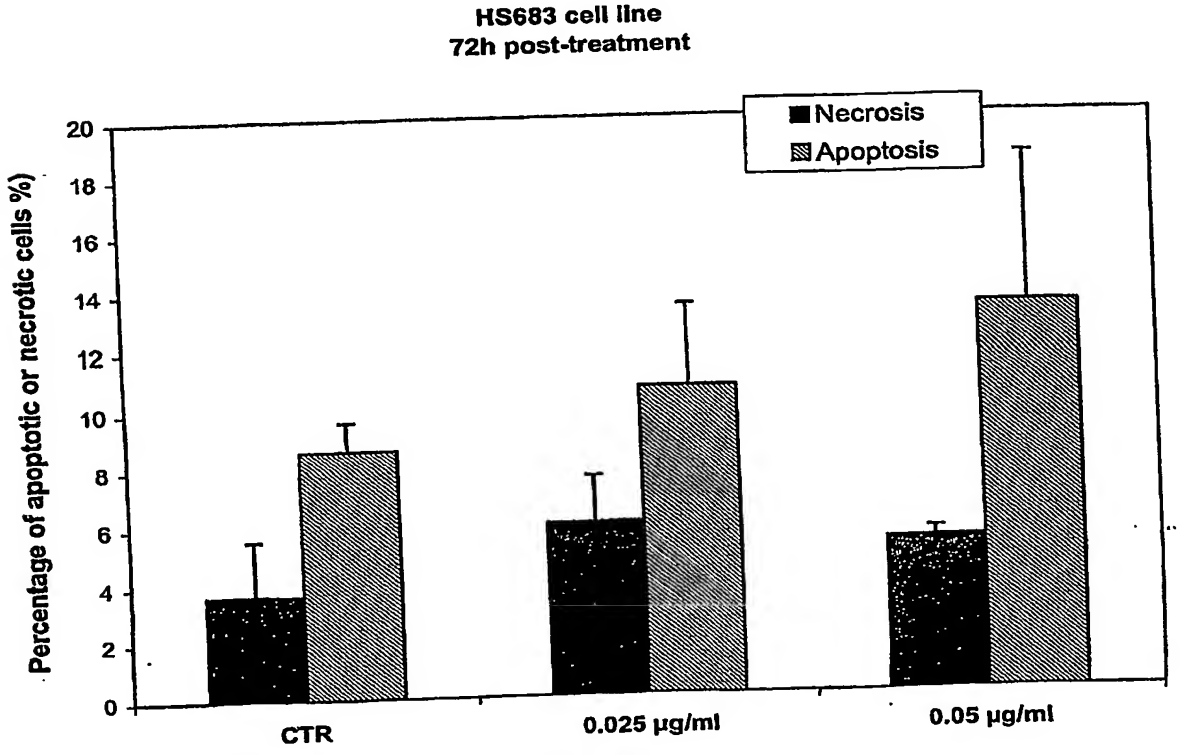
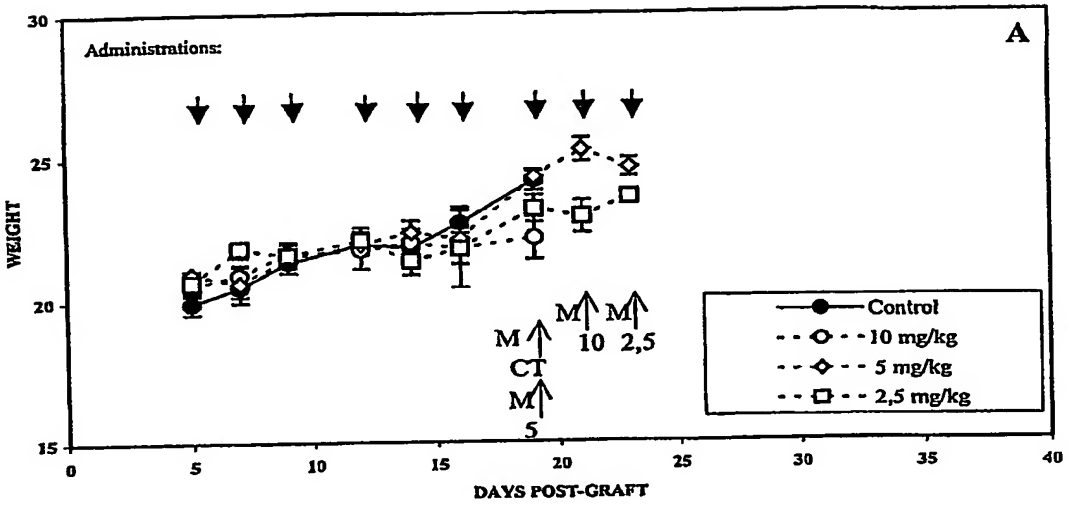

HS683 cell line
72h post-treatment
Necrosis
Apoptosis
Percentage of apoptotic or necrotic cells %)
0
2
4
6
8
10
12
14
16
18
20
CTR
0.025 µg/ml
0.05 µg/ml

FIG. 20


A
Administrations:
WEIGHT
DAYS POST-GRAFT
Control
10 mg/kg
5 mg/kg
2,5 mg/kg
M CT
M 5
M 10
M 2,5

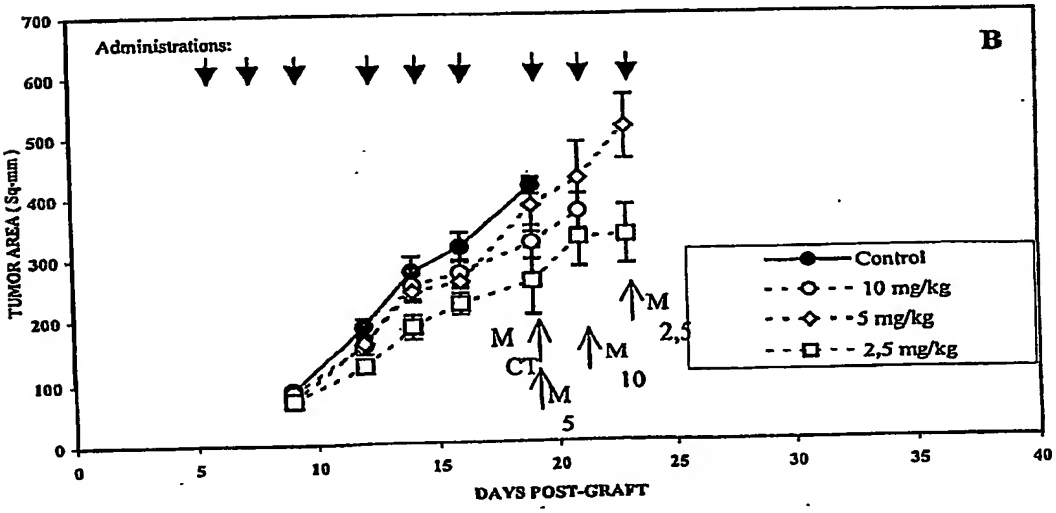

B
Administrations:
TUMOR AREA (Sq-mm)
DAYS POST-GRAFT
Control
10 mg/kg
5 mg/kg
2,5 mg/kg
M CT
M 5
M 10
M 2,5

FIG. 21

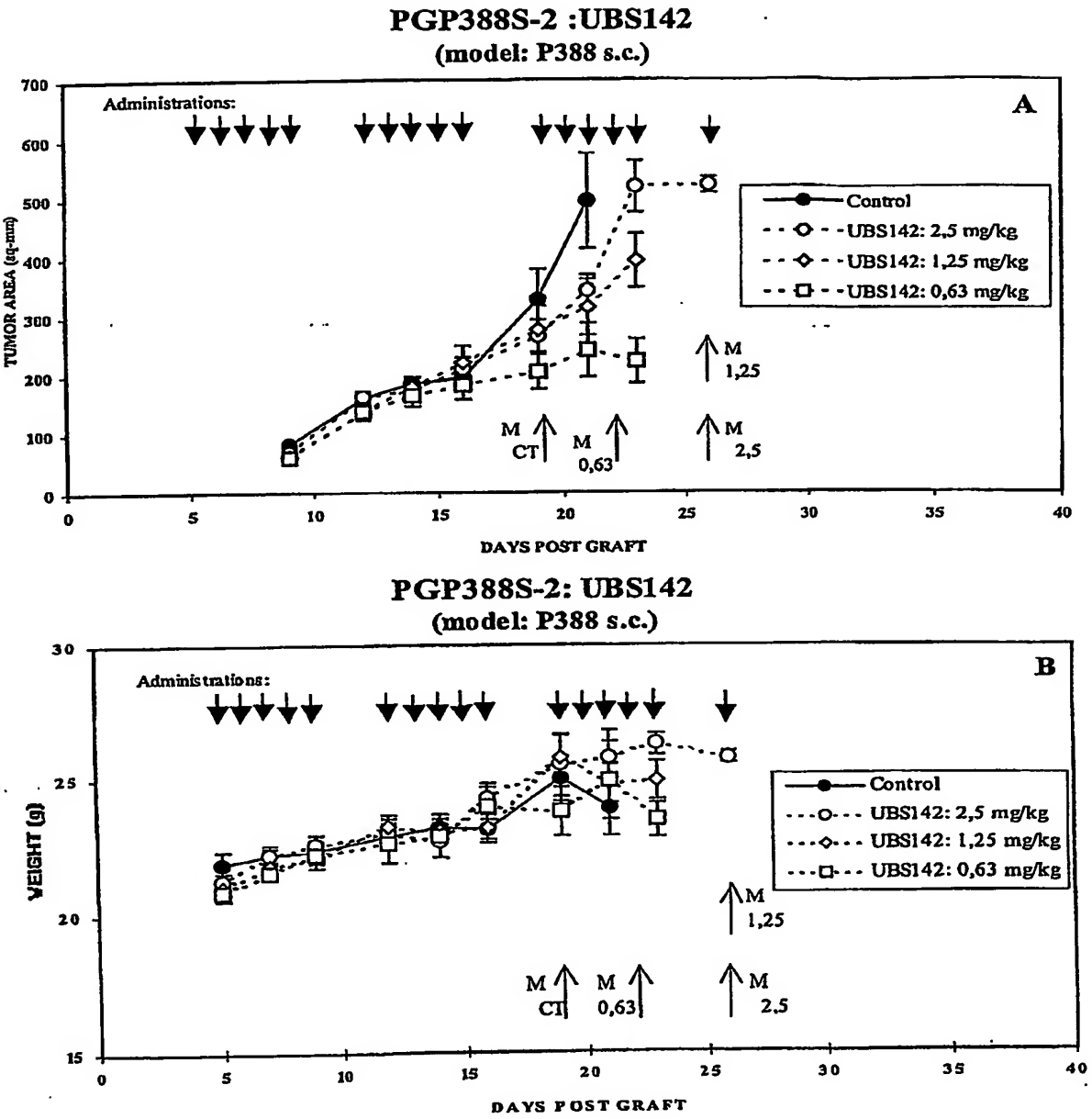
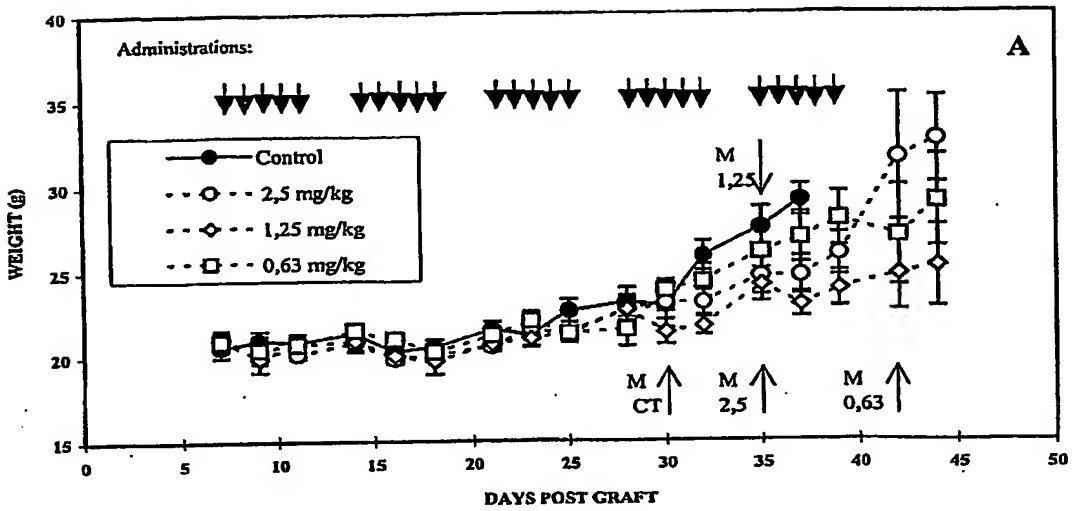

PGP388S-2 :UBS142
(model: P388 s.c.)
Administrations:
A
TUMOR AREA (sq-mm)
DAYS POST GRAFT
Control
UBS142: 2,5 mg/kg
UBS142: 1,25 mg/kg
UBS142: 0,63 mg/kg
M CT
M 0,63
M 2,5
M 1,25
PGP388S-2: UBS142
(model: P388 s.c.)
Administrations:
B
WEIGHT (g)
DAYS POST GRAFT
Control
UBS142: 2,5 mg/kg
UBS142: 1,25 mg/kg
UBS142: 0,63 mg/kg
M CT
M 0,63
M 2,5
M 1,25

FIG. 22


A
Administrations:
Control
2,5 mg/kg
1,25 mg/kg
0,63 mg/kg
WEIGHT (g)
DAYS POST GRAFT
M CT
M 2,5
M 1,25
M 0,63

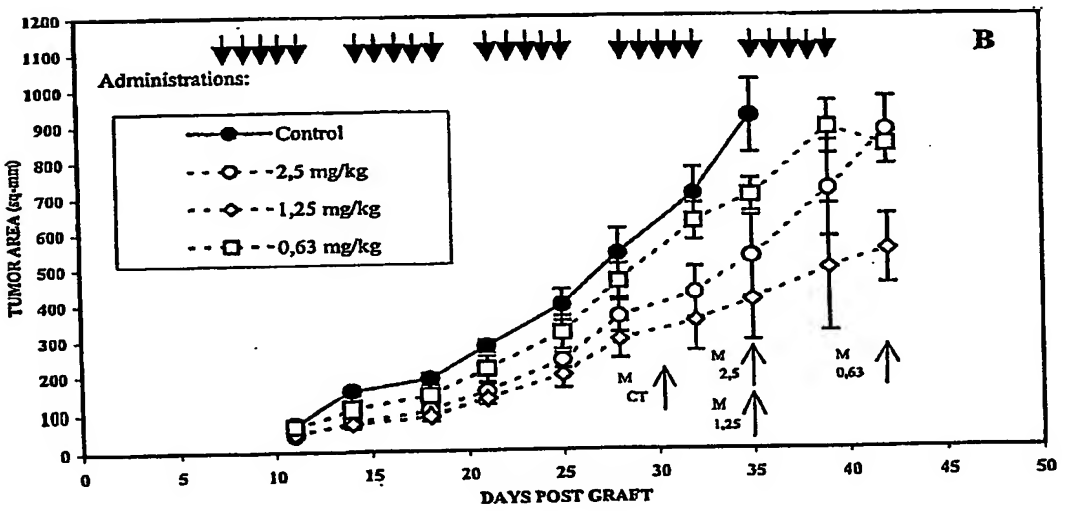
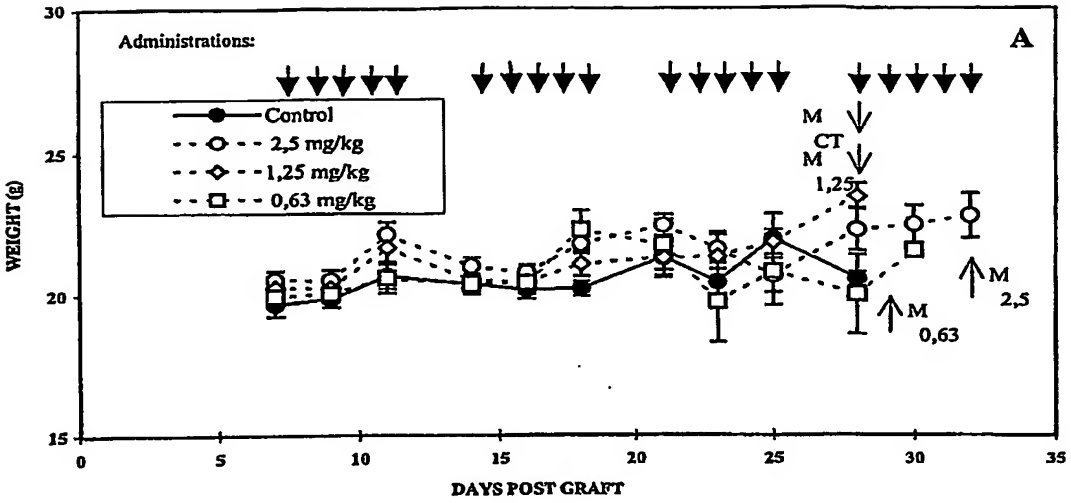

B
Administrations:
Control
2,5 mg/kg
1,25 mg/kg
0,63 mg/kg
TUMOR AREA (sq-mm)
DAYS POST GRAFT
M CT
M 2,5
M 1,25
M 0,63

FIG. 23


A
Administrations:
Control
2,5 mg/kg
1,25 mg/kg
0,63 mg/kg
WEIGHT (g)
DAYS POST GRAFT
M CT
M 1,25
M 2,5
M 0,63

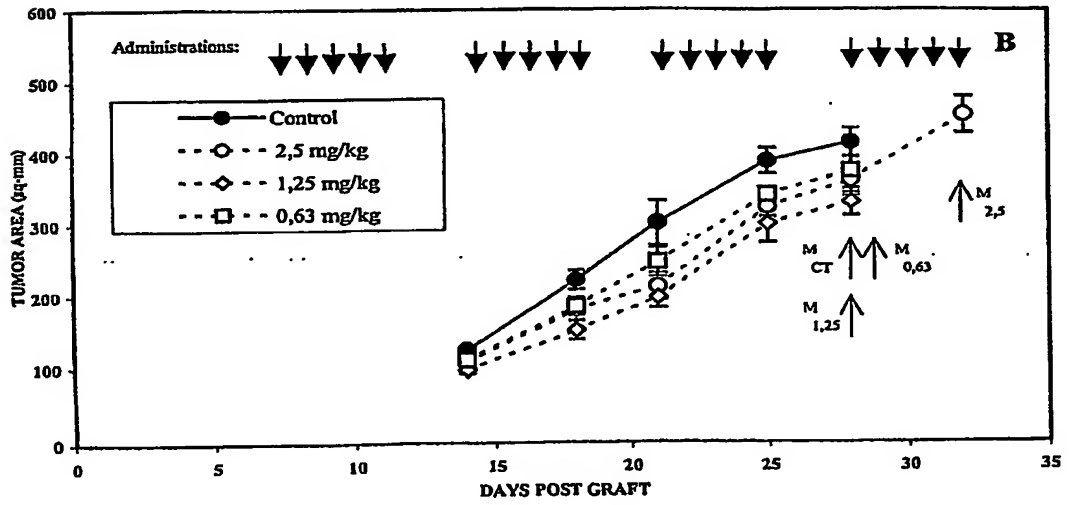

B
Administrations:
Control
2,5 mg/kg
1,25 mg/kg
0,63 mg/kg
TUMOR AREA (pq-mm)
DAYS POST GRAFT
M 2,5
M CT
M 0,63
M 1,25

FIG. 24

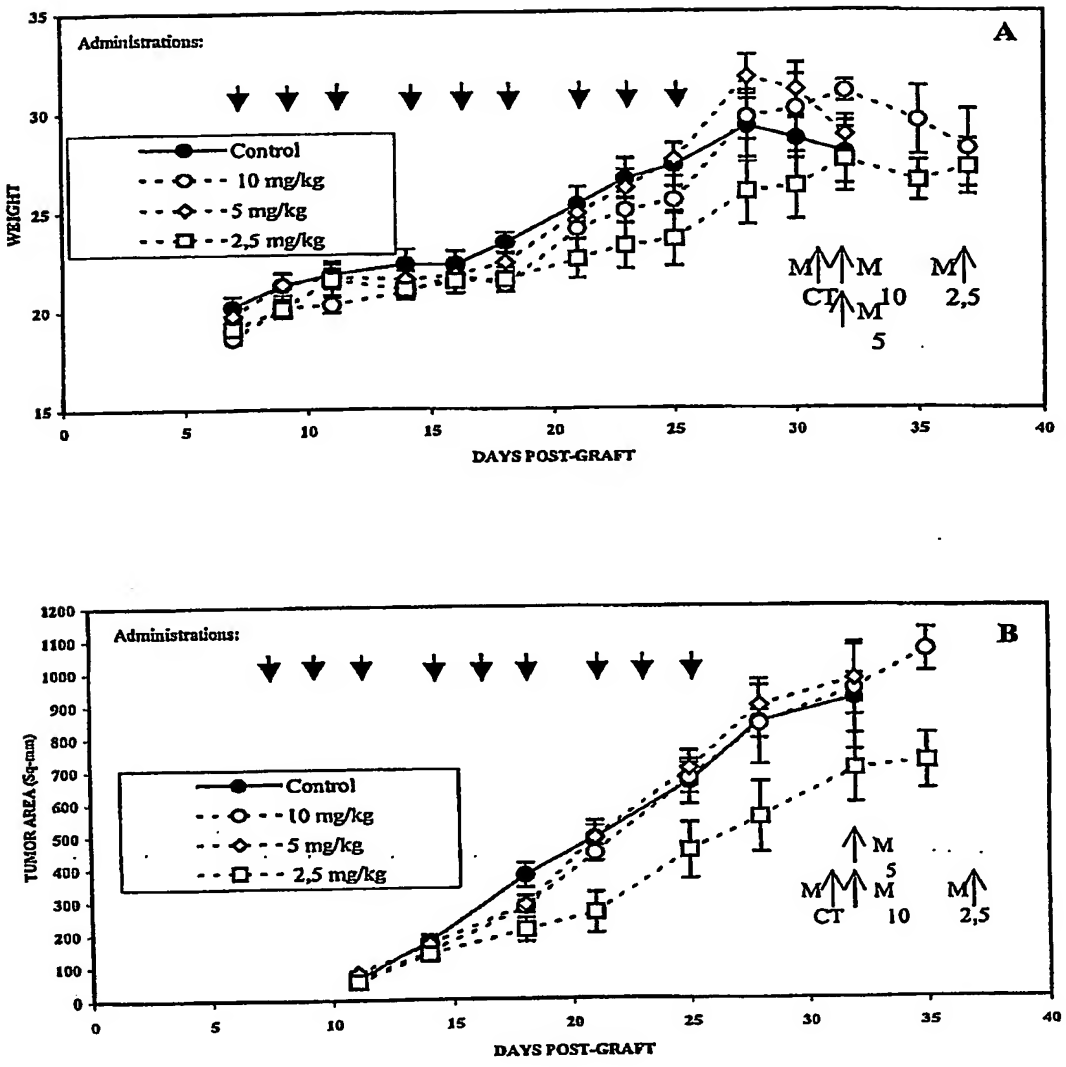

A
Administrations:
Control
10 mg/kg
5 mg/kg
2,5 mg/kg
WEIGHT
DAYS POST-GRAFT
M
CT
M
10
M
5
M
2,5
B
Administrations:
Control
10 mg/kg
5 mg/kg
2,5 mg/kg
TUMOR AREA (Sq-mm)
DAYS POST-GRAFT
M
CT
M
10
M
5
M
2,5

FIG. 25

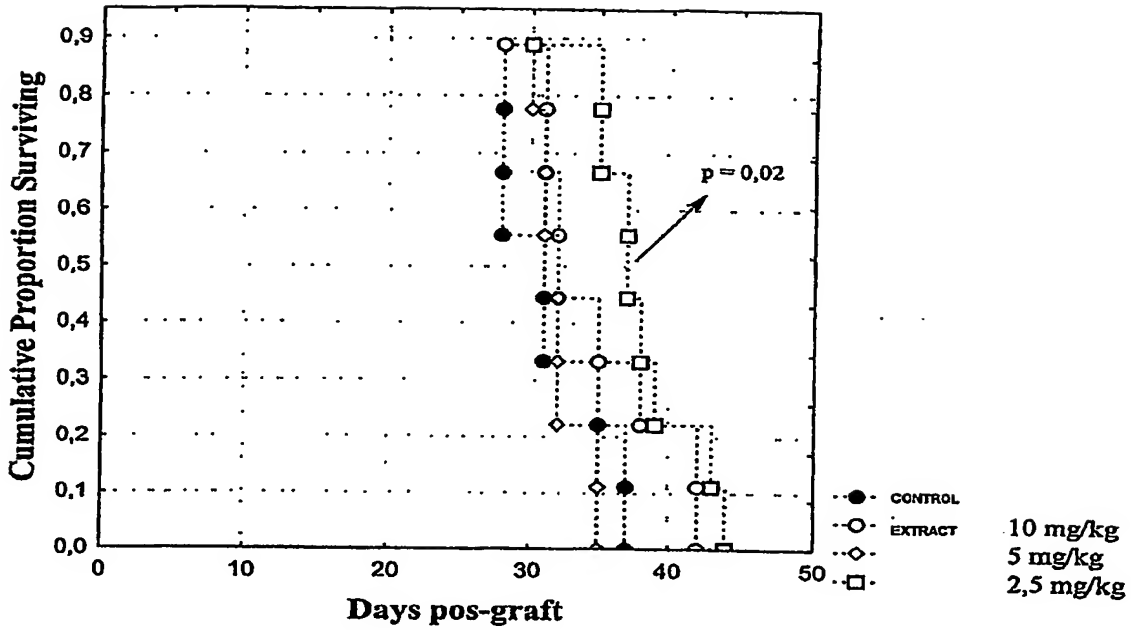

Cumulative Proportion Surviving
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0
10
20
30
40
50
Days pos-graft
p = 0,02
CONTROL
EXTRACT
10 mg/kg
5 mg/kg
2,5 mg/kg

FIG. 26

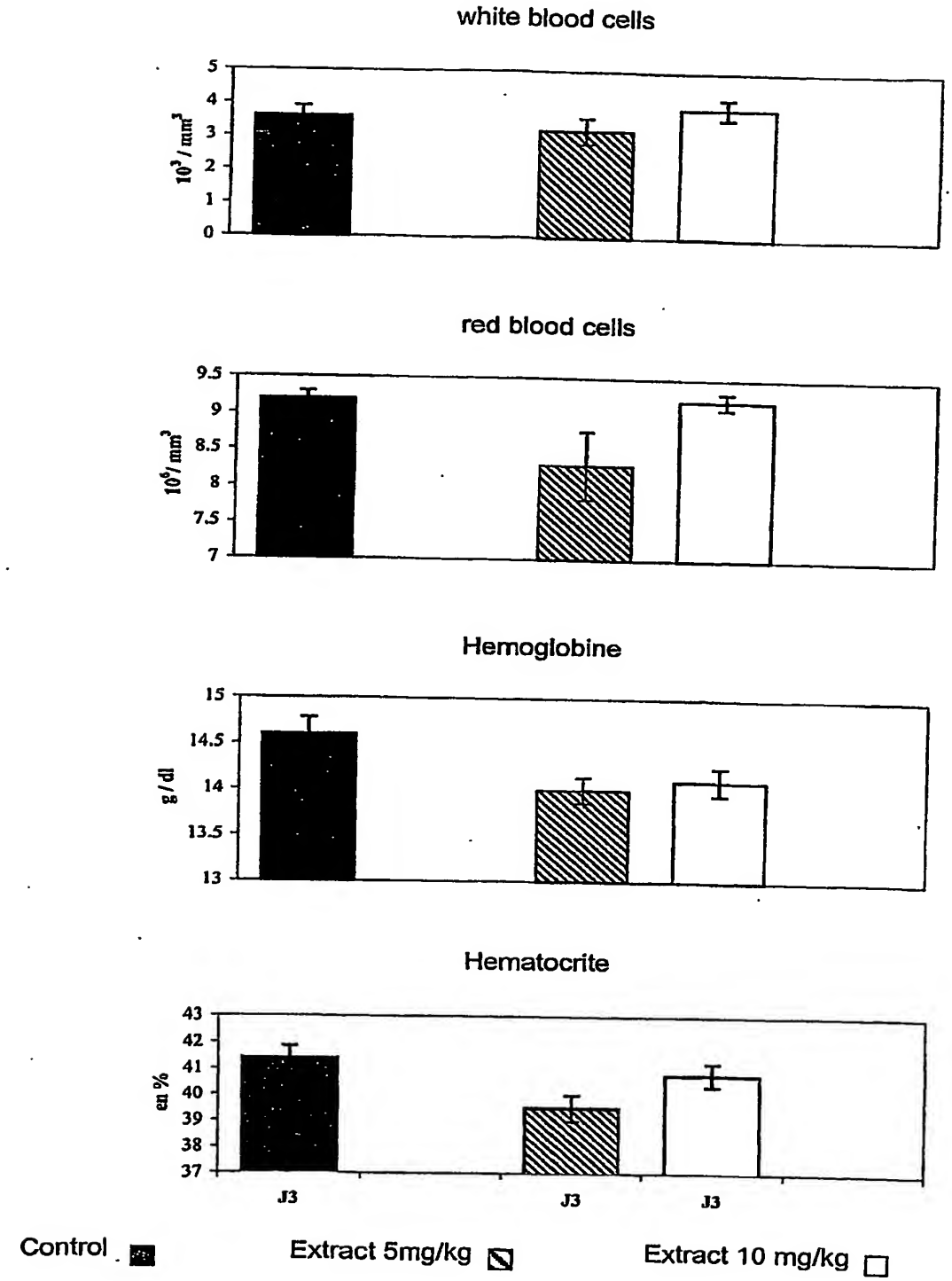
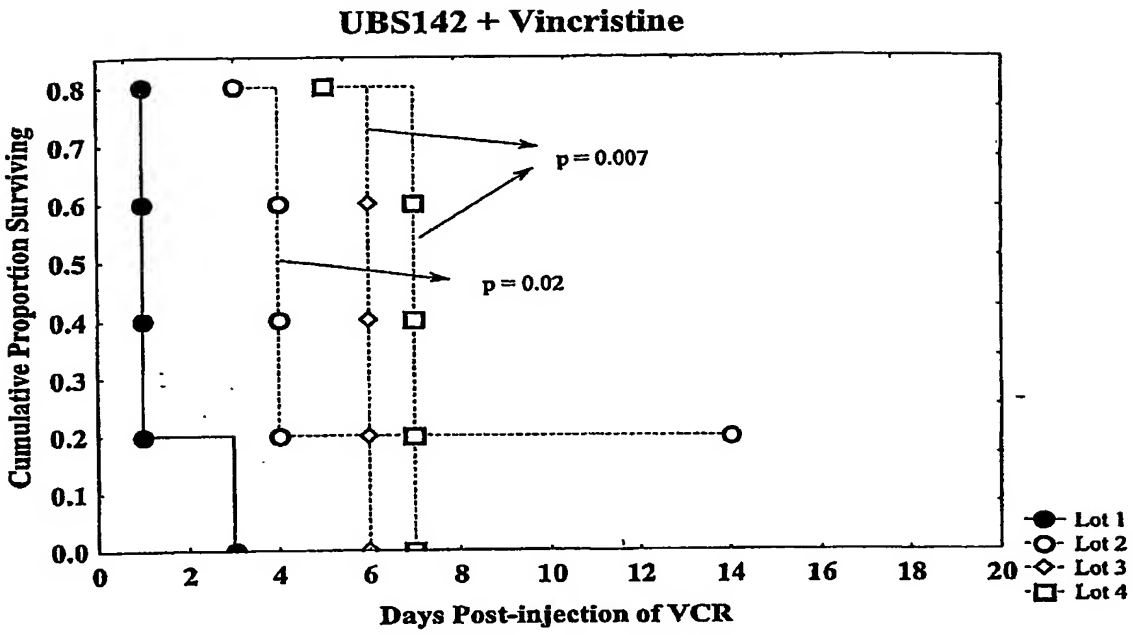

white blood cells
$10^3/mm^3$
5
4
3
2
1
0
red blood cells
$10^6/mm^3$
9.5
9
8.5
8
7.5
7
Hemoglobine
g/dl
15
14.5
14
13.5
13
Hematocrite
en %
43
42
41
40
39
38
37
J3
J3
J3
Control
Extract 5mg/kg
Extract 10 mg/kg

FIG. 27


UBS142 + Vincristine
Cumulative Proportion Surviving
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
2
4
6
8
10
12
14
16
18
20
Days Post-injection of VCR
p = 0.007
p = 0.02
Lot 1
Lot 2
Lot 3
Lot 4

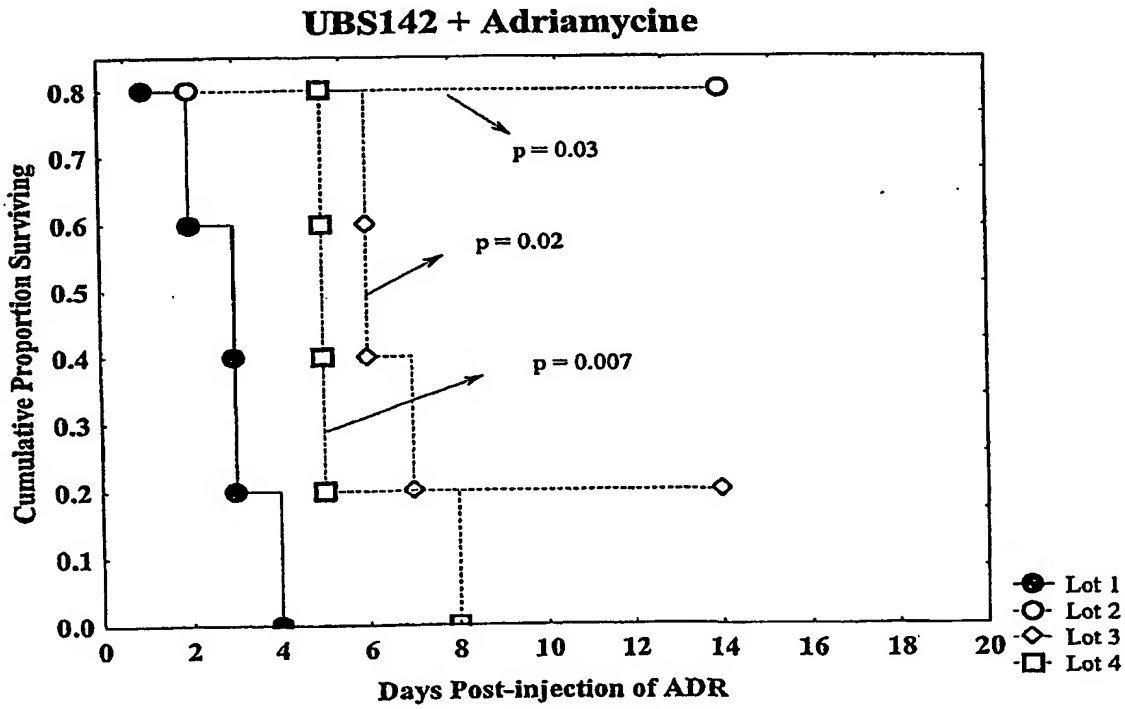

UBS142 + Adriamycine
Cumulative Proportion Surviving
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
2
4
6
8
10
12
14
16
18
20
Days Post-injection of ADR
p = 0.03
p = 0.02
p = 0.007
Lot 1
Lot 2
Lot 3
Lot 4

FIG. 28